REVIEW

For reprint orders, please contact: reprints@futuremedicine.com

Future MICROBIOLOGY

It is all about location: how to pinpoint microorganisms and their functions in multispecies biofilms

Angela M Costa[1,2,3], Filipe J Mergulhão[1], Romain Briandet[4] & Nuno F Azevedo*,[1]

Multispecies biofilms represent the dominant mode of life for the vast majority of microorganisms. Bacterial spatial localization in such biostructures governs ecological interactions between different populations and triggers the overall community functions. Here, we discuss the pros and cons of fluorescence-based techniques used to decipher bacterial species patterns in biofilms at single cell level, including fluorescence *in situ* hybridization and the use of genetically modified bacteria that express fluorescent proteins, reporting the significant improvements of those techniques. The development of tools for spatial and temporal study of multispecies biofilms will allow live imaging and spatial localization of cells in naturally occurring biofilms coupled with metabolic information, increasing insight of microbial community and the relation between its structure and functions.

First draft submitted: 21 March 2017; Accepted for publication: 9 May 2017; Published online: 26 July 2017

KEYWORDS
• biofilms • identification
• labeling • localization
• multispecies

The importance of analyzing spatial distribution in polymicrobial biofilms

Biofilms are complex and dynamic systems constituted by surface-attached bacteria embedded in a self-produced extracellular polymeric matrix [1]. This matrix is a highly hydrated structure composed of polysaccharides, proteins, glycoproteins, glycolipids and extracellular DNA and is important for biofilm surface attachment, cohesion, nutrient acquisition and protection [2]. The majority of laboratory studies are performed with single species biofilms, which are more reproducible and easier to analyze than their multispecies counterparts. While these studies provide important glimpses of how microorganisms behave in biofilms, they do not reflect what happens in real-case scenarios. In fact, it is now well accepted that the majority of naturally occurring biofilms are composed of multiple species, and many excellent reviews on this topic were published very recently [3–5]. These biofilms are a combination of different bacterial species or different microorganisms including bacteria, microalgae, protozoa or fungi that can compete for nutrients or use them synergistically in a more efficient manner.

Because polymicrobial biofilms are ubiquitous, synergistic interactions may prevail over antagonistic interactions [1,6]. The interactions between different species present in the biofilm are mediated by specific molecules such as proteins, DNA and metabolites. These interactions play a critical role in biofilm formation, in its global organization, stability, extracellular polymeric matrix composition, antibiotic resistance, virulence and ability to degrade pollutants [2,3,7–10].

[1]LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal
[2]i3S – Instituto de Investigação e Inovação em Saúde, University of Porto, Portugal
[3]INEB – Institute of Biomedical Engineering, University of Porto, Porto, Portugal
[4]Micalis Institute, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France
*Author for correspondence: nazevedo@fe.up.pt

Future Medicine part of fsg

The identification of the microorganisms present in a biofilm can be performed using methodologies that compromise the structure of the biofilm, such as qRT-PCR [11,12], next-generation sequencing [13,14] and mass spectrometry [15]. However, knowledge about the localization of the microorganisms within this complex architecture can provide crucial information to understand the underlying microbial ecology of these sessile communities and their functions. For example, in a dual-species biofilm composed of *Acinetobacter* sp. and *Pseudomonas putida*, formed in the presence of benzyl alcohol as the sole nutrient source, *Acinetobacter* sp. was found to be located in the upper layer of the biofilm closer to the nutrient source, whereas *P. putida* was localized in the deeper layers [16]. This spatial organization was attributed to a faster uptake of benzyl alcohol by *Acinetobacter* sp., which would then convert the substrate into benzoate, a compound that could in turn be used as a substrate by *P. putida*. Another example of the importance of localizing microorganisms within biofilms can be found in the study by Gantner *et al.* [17], in which authors measured the 'calling distance' between two different strains of *P. putida*, that is, the maximum distance at which two different bacterial cells can communicate through quorum sensing. This distance was determined by combining single cell fluorescent imaging with a geostatistical analysis and was estimated to be in the range of 4–78 μm.

An even more detailed and trustworthy understanding of the metabolic activity of individual populations in biofilms can be gained by coupling spatial localization techniques and metabolic activity tracking at an individual cell level. This allowed, for instance, Lucker *et al.* to establish a key role of *Nitrotoga*-like bacteria in the oxidation of nitrites in wastewater treatment plants [18]. Their conclusions were based on the agreement of two data sets: one indicating that spatially, *Nitrotoga* co-aggregated with bacteria that carried out ammonia oxidation processes and the other that showed that carbon fixation by individual cells of *Nitrotoga* was nitrite-dependent.

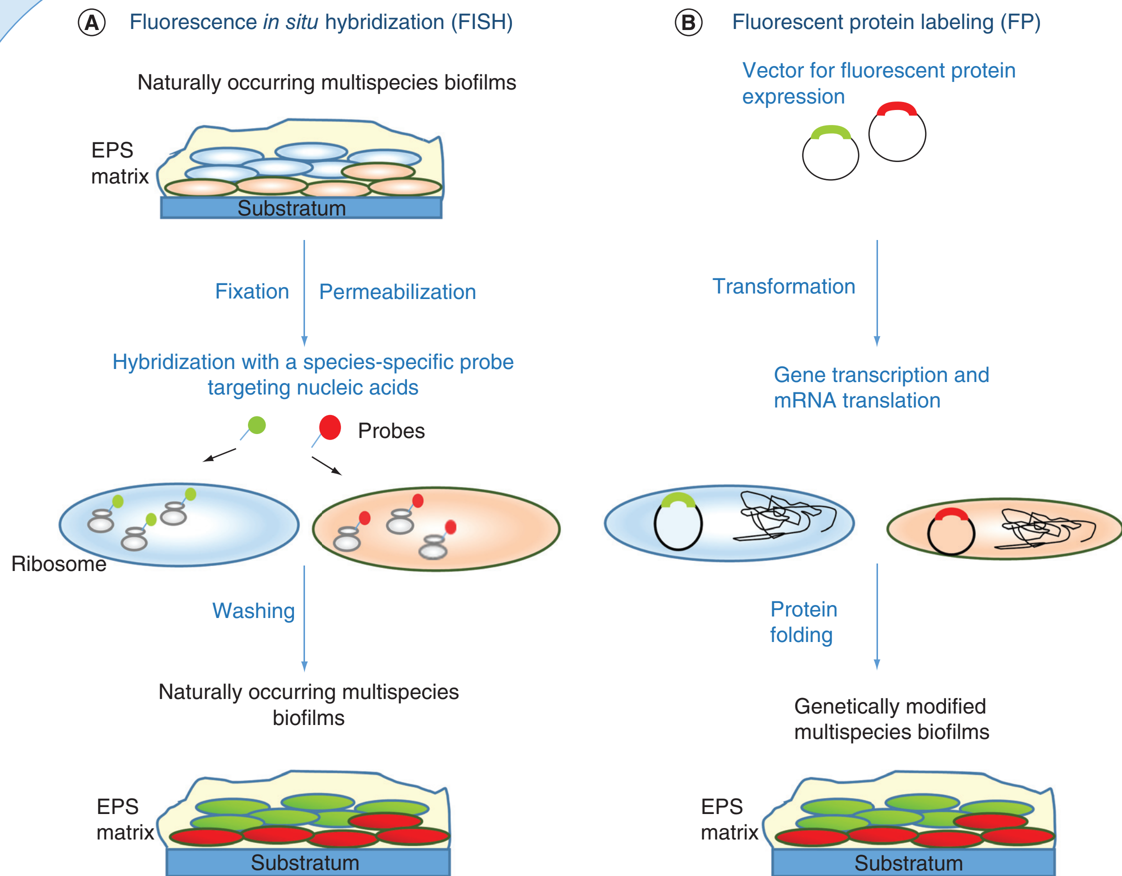
As illustrated above, biofilms are not just a geometric tridimensional assemblage of microorganisms, they have a dynamic spatial organization that responds to local environmental variations and confers unique genetic and physiological properties to their matrix cohabitants.

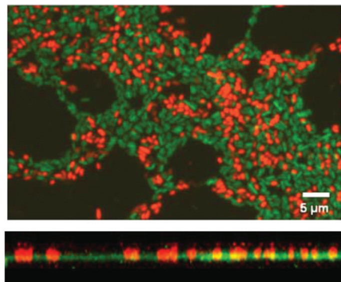
How to visualize specific microorganisms at single-cell scale in polymicrobial biofilms?

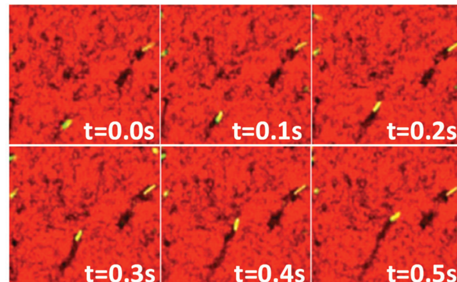
Ultrastructural microscopy techniques such as scanning electron microscopy can provide an indication of species distribution in biofilms, but solely when microorganisms are morphologically very distinct. In addition, the diversification of morphotypes for certain bacteria such as *Lactococcus lactis*, which can generate both ovoccoids and filaments in biofilms depending on the cell local microenvironment [19], prevents a more general use of these techniques. Chemical-imaging technologies, which include Raman spectroscopy, can also be a promising alternative for the mapping of fully hydrated biofilms. Nonetheless, at this time point the methodology has been only sparsely applied in the identification of microorganisms in 3D structures [20], which reflects technical limitations of the method associated with the speed of analysis and also the intensity of the Raman signal [20,21].

The large majority of techniques used to assess the spatial organization of biofilm species are based on the application of dyes or fluorochromes combined with optical microscopy. These colorimetric or fluorescent dyes vary in their ability to discriminate populations. Some dyes, such as those used in Gram-staining, allow the discrimination between Gram-positive and Gram-negative bacteria, whereas unspecific nucleic acid dyes such as DAPI and Syto™ are used for the visualization of the entire population [22]. These dyes can also be used in combination with species-specific fluorescent antibodies [23], but technical constraints such as antibody cross-reactivity, poor penetration into the biofilm matrix and the difficulty in generating appropriate antibodies for nonpathogenic strains, make this technique unpractical in most situations faced by biofilm researchers.

Arguably, two of the most powerful and widely used methods to study strains or species-specific spatial organization in biofilms are based on the labeling of microorganisms using fluorescent proteins (FP) or with fluorescently labeled nucleic acids (fluorescence *in situ* hybridization [FISH]). FP labeling and FISH have their own distinct features that imply a different utilization of these techniques by the biofilm researcher **(Figure 1)**. For instance, unlike with FP labeling, which implies genetic modifications of the microorganisms, impairing its use in natural







	FISH	FP labeling
Advantages	Applied in naturally occurring biofilms Multiplex experiments easy to perform Microorganisms not altered before experiment Rapid detection and identification of microorganisms	Non-destructive technique Biofilm formation can be followed *in vitro* It is possible to label different strains with different colors
Disadvantages	Destructive technique Probes have to diffuse into the biofilm matrix Hybridization step with well-defined conditions of temperature and pH Fluorescence dependent on the number of ribosomes	Possible loss of the vector due to selective pressure Possible poor fluorescence due to low expression levels Bacteria transformation may alter the metabolism and survival of the cells Not applicable to naturally occurring biofilms

Figure 1. Example of multispecies biofilm visualization using (A) fluorescence *in situ* hybridization or (B) fluorescent protein labeling. The most relevant advantages and disadvantages of each method are also identified. **(A)** The confocal laser scanning microscopy image of fluorescence *in situ* hybridization represents a dual-species biofilm of *Listeria monocytogenes* (green) and *Salmonella enterica* (red) formed in Tryptic Soy Broth under static conditions. **(B)** The real-time confocal laser scanning microscopy frames represent green bacilli swimmers expressing green fluorescent protein forming transitory pores in a mature biofilm of *S. aureus* (counterstained in red with a universal bacterial probe).
EPS: Extracellular polymeric matrix.
(A) Reprinted with permission from [42].

environments, FISH can be used to assess natural populations without the need of genetic manipulation of strains [5]. On the other hand, FP labeling generally does not compromise the biological activity of the biofilm as it does not require a fixation step, enabling real-time and noninvasive imaging and making it extremely useful for lab-grown biofilms [24].

FISH and FP labeling share many of the same goals when applied to biofilm research: they both strive for enhanced signal intensity, to differentiate as many subpopulations as possible in a single experiment (multiplexing) and to be applicable in a wide range of situations (different physico-chemical conditions and biofilm thicknesses) while maintaining their specificity. Additionally, they should be amenable to coupling with other techniques that provide additional spatial information about the biofilms (e.g., cell activity and other biofilm components location, together with the determination of oxygen, pH or other local physico-chemical conditions).

Nonetheless, each technique has its own specific limitations and ways to circumvent them while addressing these goals (Figure 2). These aspects will now be discussed in detail, first for FISH and then for FP labeling.

FISH

FISH is a technique that typically targets the ribosomal RNA of microorganisms using fluorescently labeled oligonucleotide probes [25]. To perform this technique, cells have, in the majority of cases, to be fixed and permeabilized in

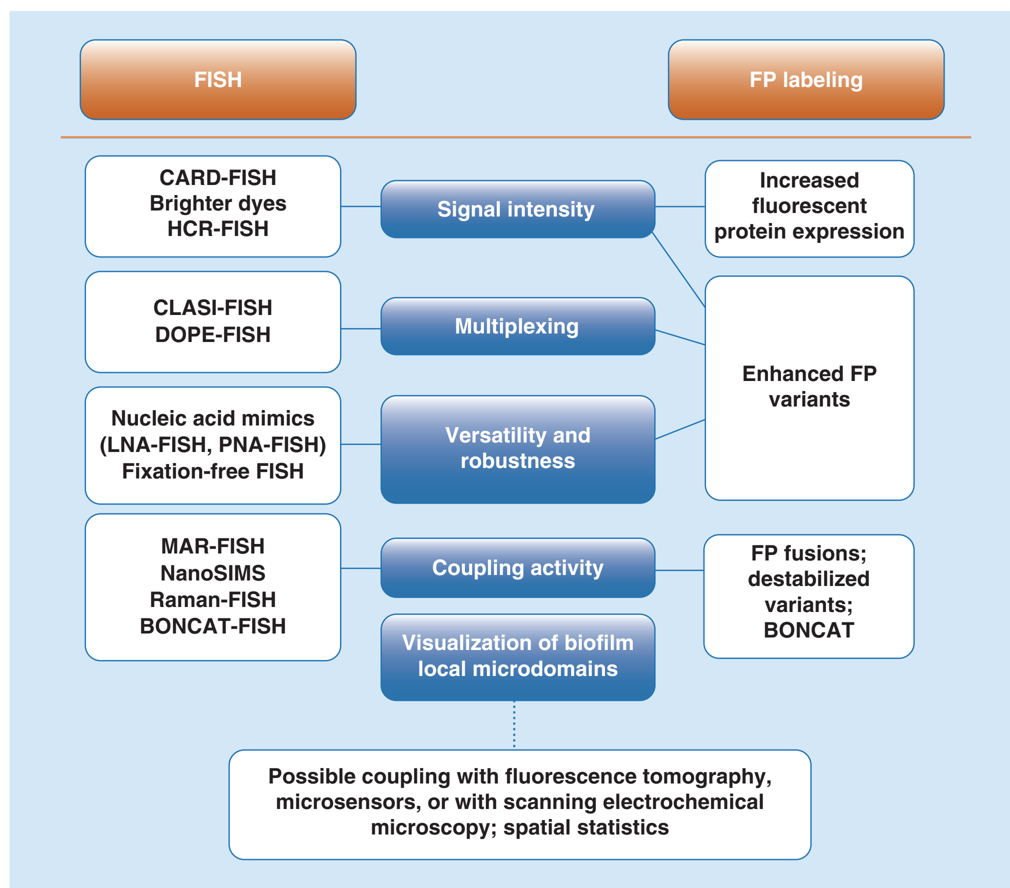


Figure 2. Limitations (blue-filled boxes) and alternatives (white boxes) to fluorescence *in situ* hybridization and fluorescent proteins labeling. The dashed line depicts a potential combination of techniques that would result in a better understanding of biofilm microdomains.
BONCAT: Bio-orthogonal non-canonical amino acid tagging; CARD: Catalyzed reporter deposition; CLASI: Combinatorial labeling and spectral imaging; DOPE: Double labeling of oligonucleotide probes; FISH: Fluorescence *in situ* hybridization; FP: Fluorescent protein; HCR: Hybridization chain reaction; LNA: Locked nucleic acid; MAR: Micro-autoradiography; NanoSIMS: Nanometer-scale secondary-ion mass spectrometry; PNA: Peptide nucleic acid.

order to allow both the maintenance of its structure and the access of the probe into the cell. Once inside the cell, the probe has to hybridize with its target (e.g., 16S rRNA), a process that occurs in well-defined conditions of pH and temperature. If hybridization occurs, the spatial location of the microorganisms in the biofilm can be analyzed, for instance, by confocal laser scanning microscopy (CLSM) [25]. When FISH started to be applied to biofilms in the early 90s, it provided the first glimpses of the relative abundance of populations in wastewater and river-water biofilms [26,27]. These studies were however limited in assessing spatial organization, as presumably the method and detection equipment (i.e., CLSM) had not been sufficiently optimized for thicker 3D biofilm structures. In addition, physiological activity was typically determined by correlating signal (fluorescence) intensity with ribosomal content, which proved for most environmental settings to be a poor indicator [28]. In the following sections, the most relevant features of FISH will be presented.

- **Signal intensity**

Microorganisms with low ribosomal content or probes targeting mRNA may account for low levels of FISH signal. To overcome this technical limitation a variation of FISH was developed, named catalyzed reporter deposition (CARD-FISH), which uses horseradish peroxidase-labeled oligonucleotide probes, in which each probe catalyzes the deposition of multiple fluorescently labeled tyramide molecules, leading to signal amplification and increased sensitivity [29]. Nonetheless, the diffusion of the protein through the biofilm brings an additional barrier to the application of CARD-FISH to thick biofilms. A simpler alternative is the use of fluorophores with higher extinction coefficients and quantum yields that will translate into brighter signals [30]. Nonetheless, this only provides a modest improvement in signal.

A new approach named double labeling of oligonucleotide probe-FISH was developed, in which a single probe is labeled with two fluorochromes, which leads to an increase of approximately twofold in the signal and avoids constrains associated with CARD-FISH such as low probe penetration [31]. Similarly, multilabeled oligonucleotide-FISH, where probes can be labeled to up to four fluorophores, was recently described as an approach to enhance signal intensity. Using as a model a marine worm that is nutritionally dependent of a consortium of different symbiont bacteria, the authors were able to demonstrate that signal intensity increased several times [32]. A more recent technique that allows improving signal intensity without the need of an enzyme for molecular amplification is the hybridization chain reaction (HCR)-FISH. HCR-FISH uses an initiator probe, with a sequence specific for the target, and in most of the cases two fluorescently labeled RNA hairpin nucleic acid probes to provide signal amplification (see [33] for more technical details). This technique was successfully used in the detection of multiple organisms in seawater, sediments and sludge samples [34,35], and in the study of the spatiotemporal gene expression patterns in a symbiotic partnership between a squid and bacteria [36]. However, in the case of Gram-positive bacteria, a permeabilization step may be still required, and the handling of the RNA probes can be problematic due to its labile nature. Regarding this last issue, more stable DNA-HCR probes were successfully tested more recently [35].

- **Multiplexing**

In classical FISH, the simultaneous visualization of different types of microorganisms is generally limited to three, in order to prevent spectral overlapping of fluorophores during microscopic analysis of the biofilms. Recently, a FISH variant named combinatorial labeling and spectral imaging (CLASI)-FISH was developed for the study of multispecies biofilms. It uses two probes with different associated fluorophores for each species, creating in that way multiple combinations of colors. After multispectral imaging analysis, the simultaneous identification and visualization of different species is possible. Using the dental plaque biofilm as a model, Valm *et al.* were able to image up to 15 different taxa simultaneously [37,38]. Nonetheless, if two different probes are directed to the same sequence within the target species, the signal intensity will be distributed by both labels, decreasing its sensitivity by half. On the other hand, if the two probes target different sites in the species of interest, probes might have different affinities to their target sequences, which will negatively affect the efficiency of hybridization. To overcome the potential loss of sensitivity due to the use of two different probes in the CLASI-FISH, double labeling of oligonucleotide probes-FISH can be used. Using multicolored, double-labeled

oligonucleotides probes to achieve multiplexing, Behnam *et al*. were able to detect up to six microorganisms in a single FISH experiment [39]. Also, the aforementioned multilabeled oligonucleotide-FISH can accurately detect up to seven different microorganisms, using multilabeled probes directed to only one site in the RNA [32]. Nonetheless, the number of different microorganisms that can be detected simultaneously by these two approaches is still significantly lower than when using CLASI-FISH.

- **Versatility & robustness**

While many of the early concerns about standard FISH have been solved and this technique is now the most widely used to discriminate microbial populations, several limitations have yet to be addressed. For a start, the need for cell fixation will presumably kill all cells in the biofilm, precluding further *in vivo* analysis, meaning that only snapshots of a biofilm can be obtained for each individual experiment. Artifacts in the biofilm structure can also occur due to a number of factors of which the most important are likely to be: sampling methodologies for naturally grown biofilms (in lab-grown biofilms analysis can be directly performed on the surface); fixation procedures, which involve changing the solvent from water to ethanol or formaldehyde; temperature increase associated to the hybridization process; and diffusion limitations of the probe through the biofilm matrix and cell envelope. The first, second and third factors may cause biofilms to deform, while the fourth leads to poor visualization of the cells, particularly those found in the inner layers of the biofilm or with thick cell walls.

To overcome part of these drawbacks, a new generation of more efficient and degradation-resistant nucleic acid mimics was developed. Examples of such mimics are peptide nucleic acid (PNA) and locked nucleic acid [40,41]. The use of PNA-FISH associated with CLSM was reported in several works that study both the multispecies biofilms composition and the spatial organization of the microorganisms, such as in a mixed biofilm of *Salmonella enterica*, *Listeria monocytogenes* and *E. coli* [42]. In this study, the probe was able to penetrate into a thick biofilm without apparent loss of signal, a feature that may be due to the uncharged nature of PNA. Locked nucleic acid-FISH probes have been used at more uncommon conditions such as acidic pH and 37°C, with the aim of detecting *H. pylori* directly in the stomach [43]. This procedure, named fluorescence *in vivo* hybridization, paves the way for direct application of FISH in real-life settings, whether they are clinical or environmental, without extensive modification of the microenvironment surrounding the biofilm. While hybridization of nonfixed cells has been also already described elsewhere [44,45], there are still many doubts regarding the cellular viability during the whole procedure and the adaptability of FISH to work in a continuous timeframe. Another positive side of this approach is that it has the potential to enable DNA analysis after FISH since the compromising step of DNA crosslinking during fixation is avoided [45].

- **Functional analysis of biofilm populations**

To assess the contribution of different microbial populations to the biofilm metabolic activity micro-autoradiography-FISH (MAR-FISH) can be used. In MAR-FISH, the metabolic activity is assessed using radioisotope-labeled substrates that are incorporated into individual cells and detected. This technique was used for example in the study of multispecies nitrifying biofilms [46]. Nevertheless, analysis of single-cell activity in complex biofilms can be complicated, especially if cells are located above each other, or mixed in clusters. Also, radioisotopes with an appropriate half-life must be used (e.g., ^{3}H, ^{14}C, ^{32}P and ^{35}S) and the technique may be unsuitable for environmental analysis. It also has the disadvantage of not being amenable to nitrogen and oxygen labeling, two major components of the organic matter. Variations of this technique that allow stable isotope-based analysis were reported and used in the study of complex microbial communities. One of these techniques is the nanometer-scale secondary-ion mass spectrometry, which uses multi-isotope imaging mass spectrometry [47,48]. Another similar technique, named Raman-FISH, combines fluorescence microscopy and Raman imaging, and can be used to demonstrate the relevance of a specific microorganism in a community. An example of its use is the work of Wagner's lab, where a combination of Raman microscopy and FISH was used to define the dominant microorganisms through the analysis of physiologically active cells [49]. This enabled the identification of microbes in the mouse cecal microbiota that respond to alterations in mucin and sugars, which in turn lead to a better understanding of the overall functions of the microbial populations [49]. In contrast

to MAR-FISH, Raman-FISH and nanometer-scale secondary-ion mass spectrometry require specialized and expensive instrumentation that is not available in the majority of the research labs. More recently, the bio-orthogonal non-canonical amino acid tagging technology was described [50]. This technique is based on the *in vivo* incorporation of the noncanonical amino acid L-azidohomoalanine (AHA), which is a surrogate for L-methionine, followed by fluorescent labeling of L-azidohomoalanine-containing cellular proteins by azide-alkyne click chemistry. The procedure can be combined with FISH for establishing a link between taxonomy and translational activity in environmental biofilms at a single-cell level. This approach, which circumvents the need for isotopic labeled samples, was successfully used in the study populations from oral microbiome, pond water and anoxic sediments [50].

Another valuable tool to relate individual organisms to their activity and biological function is mRNA-FISH, which can target the sequence of specific genes whose expression is associated with the activation of a certain metabolic pathway, providing information about the microorganism physiology. This technique was developed to work in conjugation with rRNA-FISH, and was successfully used to study the expression pattern of a gene involved in methane oxidation by aerobic methane oxidizing bacteria [51]. Since then, mRNA-FISH was used to clarify the activity and biology of several environmental microorganisms [52,53]. However, due to the nature of its target, mRNA, problems with signal intensity may exist, as the low mRNA copy number that the cell is producing at a certain time point can result in low levels of signal intensity.

FP labeling

FP labeling is based on the insertion of a gene coding for an FP in the microorganism under study. This protein will then, under certain circumstances, be expressed, and the FP detected by flow cytometry (at the single cell scale but with no spatial information), epifluorescence (2D information) or other laser-based microscopies (3D, CLSM being the most widely used technique in biofilm research). The history of FP labeling starts in the 60s when a GFP was first isolated, with pivotal steps in the late 70s with the unraveling of the chromophore structure. Bacterial FP labeling was only possible much later after the gene was successfully cloned (already in the 90s). Soon after, different FPs with different colors and enhanced properties became available, enabling higher operational flexibility and the possibility of multiplexing. If each specific species is labeled with a different color, the formation of the mixed biofilm can be followed noninvasively with real-time CLSM. Those approaches demonstrated pattern differentiation during biofilm formation [54–57] and cooperation or competition between populations [58]. Based on real-time CLSM time series of biofilms formed with FP reporter strains, Bridier *et al.* proposed a new model-based method to detect interspecific interactions during biofilm development [59]. In the following sections, the most relevant features of FP labeling will be presented.

- **Signal intensity**

In FP labeling, higher signal intensity can be obtained both from the biological specimen, by using enhanced variants of FPs, and/or by increasing the FP expression in the cells [60]. Increasing FP expression can be obtained by careful design of the expression vector, which can be achieved by choosing the appropriate promoter and gene dosage or by performing codon optimization among other strategies [61]. Other concerns in FP labeling are related to the photostability of the protein, as all FPs eventually photobleach after extended excitation time. Other problems are associated with protein oligomerization and toxicity, as original FPs needed to oligomerize and tetrameric versions may be toxic to some cells. Additionally, the fluorescence yield of FPs is severely affected by their physico-chemical environment (pH, oxygen content) [30]. The use of optimized GFP variants can tackle most of those limitations [60], but these variants must be carefully selected to address the specific challenges in each application.

- **Multiplexing**

Despite the comprehensive list of FP spectral variants that are currently available [60], multi-species FP labeling experiments typically involve less than three colored populations to ensure sufficient signal to noise ratio for single cell scale resolution. However, using the right filter settings on CLSM, and combining each target with two FPs, would enable the detection of more populations. This raises further concerns related to protein expression limitations (transcription and translational limitations and protein stability) and the increased metabolic burden for the host strain with severe impact on its ‘natural’ physiology [61].

- **Versatility & robustness**

Arguably, the main disadvantage of FP labeling is not being suitable for analysis of naturally occurring biofilms. Because this approach involves genetic modification of the organisms, this methodology can also affect bacterial behavior and biofilm properties, for instance when expression plasmids are used [62]. The use of fluorescently labeled proteins requires oxygen for protein maturation, which can also be a limiting factor in the case of dense or anaerobic biofilms [63,64]. However, maturation of some FPs is accomplished at low oxygen concentrations enabling fluorescence measurements after the samples have been exposed to air for brief periods [60]. Efficient expression of FPs is also pH dependent and highly limited in strongly acidic environments (pH below 5). This can become problematic with biofilms involving lactic acid bacteria and other acid producers [64,65], although the use of a buffered growth media may be sufficient to overcome this limitation [66]. In addition, genes expressing the FP are frequently inserted on plasmids. This has potential limitations such as plasmid stability, homogeneity of expression within the population and the need to maintain antibiotic selection pressure in the strains [67–69]. Most of those limitations can be overcome by integration of the FP genes in the chromosome, which is most often more laborious than working with plasmids [61]. Another alternative is the use of the above discussed bioorthogonal noncanonical amino acid tagging technology, which is suitable for environmental microorganism species analysis, in a pH, O_2, and temperature independent manner [50].

- **Functional analysis of biofilm populations**

FP-labeled strains reporting gene expression in biofilms enable visualization of specific spatiotemporal patterns of expression in these organized communities. These approaches contributed to the discrimination of cell types in biofilms that were linked to specific biofilm behaviors, such as tolerance to stresses and antimicrobials [70,71]. FP-labeled strains were used to demonstrate that some genes were only expressed in certain regions and at certain stages of biofilm formation [72–75]. Multiplexing fluorescent reporter systems in *Bacillus subtilis* demonstrated the stratification of different cell phenotypes expressing flagella, matrix-producing and sporulating cells, in complex colony biofilm models [76,77]. Another interesting possibility is to use FPs for dynamic gene expression studies. In this case, dynamic measurements are often hampered by the high stability of GFP and therefore the use of destabilized GFP variants is a possibility [78].

Visualization of biofilm local microdomains

Besides the identification of the microbial species present in a biofilm and the assessment of their spatial organization, there are several other parameters that can be analyzed locally, such as differences in matrix composition, pH, chemical species distribution and their oxidative state, coadhesion strength and oxygen tension [79]. The analysis of these parameters can generate valuable data to obtain a more complete picture of the biofilm behavior. Soon after FISH was developed, the technique was used in conjugation with O_2 and NO_3^- microsensors in the study of nitrifying biofilms, which enabled the identification of the profile of ammonia and nitrite-oxidizing bacteria in the biofilm [80]. These analyses are performed sequentially in the same biofilm, and are hence unable to provide exact spatial correspondence between both sets of data. In fact, at this point there are not many examples of robust and simultaneous observation of these technologies with either FISH or FP labeling. Nonetheless, some techniques, such as the use of fluorescently labeled silica nanoparticle sensors detected by fluorescence tomography (to assess pH gradients) [81], require fluorescence-based equipment for detection and as such are likely amenable to coupling, as long as care is taken in the selection of the correct fluorophores. On the other hand, scanning electrochemical microscopy has been used to assess the local concentrations of certain molecular species, such as hydrogen peroxide and the redox-active signaling molecule pyocyanin [82,83]. However, because the detection method is based on an electrochemical detector, coupling with FISH or FP labeling is likely to be more complex. Other nondestructive techniques that can be used to assess differences in chemical composition and that might be amenable to coupling are synchrotron radiation-based Fourier transforms infrared microspectroscopy [84], Raman spectroscopy [85,86] and MALDI-TOF-FISH [87].

Under the context of the spatial distribution of biofilm components, software that can be combined with either FISH or FP imaging is of crucial importance. Different solutions are available for this purpose. A telling example is the digital image analysis in microbial ecology, which can quantify microbial co-localization,

determine whether the pairwise arrangement of the analyzed microorganisms is random, attractive or repulsive, and correlate species localization with physiological functions or physico-chemical microdomains [88,89].

Conclusion & future perspective

The vast majority of biofilms are multispecies, and given their importance in the clinical, industrial and environmental contexts, it is crucial to obtain a deeper characterization of these biostructures in all their relevant aspects. FISH and FP labeling, the most relevant techniques enabling the identification and spatial localization of microorganisms in multispecies biofilms, share a number of common features (multiplexing, versatility and robustness, among many others). Due to the specific aspects of each technique, the strategies to improve their performance differ with the application under study. For instance, FP labeling is more adequate for studying dynamic events (such as those involved in the initial adhesion and biofilm formation), whereas biofilms with more than five species will likely require a variant of the FISH-derived technique, such as CLASI-FISH.

EXECUTIVE SUMMARY

The importance of studying multispecies biofilms

- The majority of naturally occurring biofilms are multispecies.
- Both the composition and organization of the multispecies biofilms impact on the entire biofilm behavior.

Major fluorescent-based techniques to identify & study multispecies biofilm spatial organization

- Fluorescence *in situ* hybridization (FISH).
- Genetically modified fluorescent bacteria.

Major features associated with improvement of multispecies biofilms study techniques

- Signal intensity:
  - FISH: Catalyzed reporter deposition-FISH, double labeling of oligonucleotide probe-FISH, multilabeled oligonucleotide-FISH, hybridization chain reaction-FISH.
  - Fluorescent protein (FP) labeling: use of enhanced variants of fluorescent proteins, and increased expression of FP in cells.
- Multiplexing:
  - FISH: Combinatorial labeling and spectral imaging-FISH, double labeling of oligonucleotide probe-FISH, multilabeled oligonucleotide-FISH.
  - FP labeling: Careful choice of microscopic fluorescent filter settings combining each target with two FPs.
- Versatility and robustness:
  - FISH: Peptide nucleic acid-FISH, locked nucleic acid-FISH.
  - FP labeling: Bio-orthogonal noncanonical amino acid tagging.
- Functional analysis of biofilm populations:
  - FISH: Micro-autoradiography-FISH, nanometer-scale secondary-ion mass spectrometry, Raman-FISH, bio-orthogonal noncanonical amino acid tagging, mRNA-FISH.
  - FP labeling: Dynamic gene expression studies.

Future perspective

- Use of fluorescence *in vivo* hybridization to multispecies biofilm study.
- General development of fixation-free FISH techniques.
- Use of high resolution fluorescent microscopy.
- Probes transmitted through different microbial generations.
- Combined use of FISH and FP labeling.

It will be interesting to observe how each technology will cope with its specific limitations in the future and tries to acquire some of the positive aspects of the other. As an example, and as discussed above, FISH is being explored to enable the *in vivo* observation of biofilms and overcome the fixation step. In fact, there are very recent reports demonstrating that *H. pylori* can be labeled directly within the mice stomach using nucleic acid mimics in combination with FISH [90]. For the detection of the labeling, stomachs had to be collected for observation *ex vivo* under standard epifluorescence microscopy, because the sensitivity of confocal laser endomicroscopy was not sufficient for *in vivo* detection. Overcoming current limitations will involve improvements not only on the FISH methodology, to include better probe delivery in the absence of fixation, but also at the level of the endomicroscopy equipment. As another example, FP labeling can now be used to analyze patterns of gene expression within the biofilm with single cell resolution, establishing the connection between cellular activity and species identification, albeit in non-natural environments. FP labeling can also benefit from the emergence of high resolution microscopy that enables the combination of species identification with subcellular localization [91].

A longer-term goal of both techniques would be to provide a robust, highly multiplexed and activity-coupled strategy to detect live microorganisms directly in naturally occurring biofilms (i.e., *in vivo*) and with time. Ideally, probes would be transmitted through different microbial generations, providing information of biofilm dynamics at longer time scales. In the meantime, a more encompassing strategy for the spatial characterization of polymicrobial biofilms requires the combined use of both FISH and FP labeling. It is nonetheless desirable that a single unified methodology or alternative methodologies could emerge in the near future, in order to better unravel the complexity of these fascinating microbial structures.

Acknowledgements

The authors would like to acknowledge networking support by the COST Action FA1202. AM Costa was supported by the European Research Project SusClean (FP7-KBBE-2011-5, project number: 287514), and by the FCT fellowship SFRH/BPD/109446/2015. The authors are solely responsible for this work. J Deschamps and the INRA MIMA2 imaging platform are acknowledged for real-time CLSM images.

Disclaimer

The views expressed in this review do not represent the opinion of the Community, and the Community is not responsible for any use that might be made of data appearing herein.

Financial & competing interests disclosure

This work was financially supported by: Project UID/EQU/00511/2013-LEPABE, by the FCT/MEC with national funds and when applicable co-funded by FEDER in the scope of the P2020 Partnership Agreement; Project NORTE-07–0124-FEDER-000025 – RL2_ Environment & Health, by FEDER funds through 'Programa Operacional Factores de Competitividade – COMPETE', by the 'Programa Operacional do Norte' (ON2) program and by national funds through FCT – Fundação para a Ciência e a Tecnologia (DNAmimics Research Project PIC/IC/82815/2007). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1. Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* 2(2), 95–108 (2004).
2. Flemming HC, Wingender J. The biofilm matrix. *Nat. Rev. Microbiol.* 8(9), 623–633 (2010).
3. Elias S, Banin E. Multi-species biofilms: living with friendly neighbors. *FEMS Microbiol. Rev.* 36(5), 990–1004 (2012).
4. Short FL, Murdoch SL, Ryan RP. Polybacterial human disease: the ills of social networking. *Trends Microbiol.* 22(9), 508–516 (2014).
5. Roder HL, Sorensen SJ, Burmolle M. Studying bacterial multispecies biofilms: where to start? *Trends Microbiol.* 24(6), 503–513 (2016).
6. Costerton JW, Cheng KJ, Geesey GG *et al.* Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.* 41, 435–464 (1987).
7. Burmolle M, Ren D, Bjarnsholt T, Sorensen SJ. Interactions in multispecies biofilms: do they actually matter? *Trends Microbiol.* 22(2), 84–91 (2014).
8. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection. *Proc. Natl Acad. Sci. USA* 110(3), 1059–1064 (2013).
9. Fazli M, Bjarnsholt T, Kirketerp-Moller K *et al.* Nonrandom distribution of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in chronic wounds. *J. Clin. Microbiol.* 47(12), 4084–4089 (2009).
10. Sanchez-Vizuete P, Orgaz B, Aymerich S, Le Coq D, Briandet R. Pathogens protection against the action of disinfectants in multispecies biofilms. *Front. Microbiol.* 6, 705 (2015).

11 Ren D, Madsen JS, Sorensen SJ, Burmolle M. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. *ISME J.* 9(1), 81–89 (2015).

12 Ren D, Madsen JS, de La Cruz-Perera CI, Bergmark L, Sorensen SJ, Burmolle M. High-throughput screening of multispecies biofilm formation and quantitative PCR-based assessment of individual species proportions, useful for exploring interspecific bacterial interactions. *Microb. Ecol.* 68(1), 146–154 (2014).

13 Han A, Zenilman JM, Melendez JH *et al.* The importance of a multifaceted approach to characterizing the microbial flora of chronic wounds. *Wound Repair Regen.* 19(5), 532–541 (2011).

14 Jones DS, Albrecht HL, Dawson KS *et al.* Community genomic analysis of an extremely acidophilic sulfur-oxidizing biofilm. *ISME J.* 6(1), 158–170 (2012).

15 Ecker DJ, Sampath R, Massire C *et al.* Ibis T5000: a universal biosensor approach for microbiology. *Nat. Rev. Microbiol.* 6(7), 553–558 (2008).

16 Christensen BB, Haagensen JA, Heydorn A, Molin S. Metabolic commensalism and competition in a two-species microbial consortium. *Appl. Environ. Microbiol.* 68(5), 2495–2502 (2002).

17 Gantner S, Schmid M, Durr C *et al. In situ* quantitation of the spatial scale of calling distances and population density-independent N-acylhomoserine lactone-mediated communication by rhizobacteria colonized on plant roots. *FEMS Microbiol. Ecol.* 56(2), 188–194 (2006).

18 Lucker S, Schwarz J, Gruber-Dorninger C, Spieck E, Wagner M, Daims H. Nitrotoga-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. *ISME J.* 9(3), 708–720 (2015).

19 Perez-Nunez D, Briandet R, David B *et al.* A new morphogenesis pathway in bacteria: unbalanced activity of cell wall synthesis machineries leads to coccus-to-rod transition and filamentation in ovococci. *Mol. Microbiol.* 79(3), 759–771 (2011).

20 Beier BD, Quivey RG, Berger AJ. Raman microspectroscopy for species identification and mapping within bacterial biofilms. *AMB Express* 2(1), 35 (2012).

21 Fischer M, Triggs GJ, Krauss TF. Optical sensing of microbial life on surfaces. *Appl. Environ. Microbiol.* 82(5), 1362–1371 (2015).

22 Neu TR, Manz B, Volke F, Dynes JJ, Hitchcock AP, Lawrence JR. Advanced imaging techniques for assessment of structure, composition and function in biofilm systems. *FEMS Microbiol. Ecol.* 72(1), 1–21 (2010).

23 Periasamy S, Kolenbrander PE. Aggregatibacter actinomycetemcomitans builds mutualistic biofilm communities with *Fusobacterium nucleatum* and *Veillonella* species in saliva. *Infect. Immun.* 77(9), 3542–3551 (2009).

24 Houry A, Gohar M, Deschamps J *et al.* Bacterial swimmers that infiltrate and take over the biofilm matrix. *Proc. Natl Acad. Sci. USA* 109(32), 13088–13093 (2012).

25 Amann RI, Krumholz L, Stahl DA. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. *J. Bacteriol.* 172(2), 762–770 (1990).

26 Poulsen LK, Ballard G, Stahl DA. Use of rRNA fluorescence *in situ* hybridization for measuring the activity of single cells in young and established biofilms. *Appl. Environ. Microbiol.* 59(5), 1354–1360 (1993).

27 Amann RI, Stromley J, Devereux R, Key R, Stahl DA. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* 58(2), 614–623 (1992).

28 Odaa Y, Slagmana S, Meijerb WG, Forneya LJ, Gottschala JC. Influence of growth rate and starvation on fluorescent *in situ* hybridization of Rhodopseudomonas palustris. *FEMS Microbiol. Ecol.* 32(3), 205–213 (2000).

29 Pernthaler A, Pernthaler J, Amann R. Fluorescence *in situ* hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl. Environ. Microbiol.* 68(6), 3094–3101 (2002).

• **Describes the use of catalyzed reporter deposition-fluorescence *in situ* hybridization (FISH) to increase signal intensity in cells with low rRNA content.**

30 Fernandez-Suarez M, Ting AY. Fluorescent probes for super-resolution imaging in living cells. *Nat. Rev. Mol. Cell Biol.* 9(12), 929–943 (2008).

31 Stoecker K, Dorninger C, Daims H, Wagner M. Double labeling of oligonucleotide probes for fluorescence *in situ* hybridization (DOPE-FISH) improves signal intensity and increases rRNA accessibility. *Appl. Environ. Microbiol.* 76(3), 922–926 (2010).

32 Schimak MP, Kleiner M, Wetzel S, Liebeke M, Dubilier N, Fuchs BM. MiL-FISH: multilabeled oligonucleotides for fluorescence *in situ* hybridization improve visualization of bacterial cells. *Appl. Environ. Microbiol.* 82(1), 62–70 (2016).

33 Choi HM, Chang JY, Trinh Le A, Padilla JE, Fraser SE, Pierce NA. Programmable *in situ* amplification for multiplexed imaging of mRNA expression. *Nat. Biotechnol.* 28(11), 1208–1212 (2010).

34 Yamaguchi T, Fuchs BM, Amann R, Kawakami S, Kubota K, Hatamoto M. Rapid and sensitive identification of marine bacteria by an improved *in situ* DNA hybridization chain reaction (quickHCR-FISH). *Syst. Appl. Microbiol.* 38(6), 400–405 (2015).

35 Yamaguchi T, Kawakami S, Hatamoto M *et al. In situ* DNA-hybridization chain reaction (HCR): a facilitated *in situ* HCR system for the detection of environmental microorganisms. *Environ. Microbiol.* 17(7), 2532–2541 (2015).

• **Use of hybridization chain reaction-FISH to improve signal intensity in analysis of environmental microorganisms.**

36 Nikolakakis K, Lehnert E, Mcfall-Ngai MJ, Ruby EG. Use of hybridization chain reaction-fluorescent *in situ* hybridization to track gene expression by both partners during initiation of symbiosis. *Appl. Environ. Microbiol.* 81(14), 4728–4735 (2015).

37 Valm AM, Mark Welch JL, Rieken CW *et al.* Systems-level analysis of microbial community organization through combinatorial labeling and spectral imaging. *Proc. Natl Acad. Sci. USA* 108(10), 4152–4157 (2011).

•• **A seminal paper that provides the first description of combinatorial labeling and spectral imaging-FISH, a technique that allows to improve significantly the number of different microorganisms that can be detected simultaneously.**

38 Mark Welch JL, Rossetti BJ, Rieken CW, Dewhirst FE, Borisy GG. Biogeography of a human oral microbiome at the micron scale. *Proc. Natl Acad. Sci. USA* 113(6), E791–E800 (2016).

39 Behnam F, Vilcinskas A, Wagner M, Stoecker K. A straightforward DOPE (double labeling of oligonucleotide probes)-FISH (fluorescence *in situ* hybridization) method for simultaneous multicolor detection of six microbial populations. *Appl. Environ. Microbiol.* 78(15), 5138–5142 (2012).

40 Silahtaroglu AN, Tommerup N, Vissing H. FISHing with locked nucleic acids (LNA): evaluation of different LNA/DNA mixmers. *Mol. Cell. Probes* 17(4), 165–169 (2003).

41 Stender H, Fiandaca M, Hyldig-Nielsen JJ, Coull J. PNA for rapid microbiology. *J. Microbiol. Methods* 48(1), 1–17 (2002).

42 Almeida C, Azevedo NF, Santos S, Keevil CW, Vieira MJ. Discriminating multi-species populations in biofilms with peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH). *PLoS ONE* 6(3), e14786 (2011).

- **Use of *in vivo* hybridization for the detection of a pathogenic microorganism.**

43 Fontenete S, Guimaraes N, Leite M, Figueiredo C, Wengel J, Filipe Azevedo N. Hybridization-based detection of *Helicobacter pylori* at human body temperature using advanced locked nucleic acid (LNA) probes. *PLoS ONE* 8(11), e81230 (2013).

44 Silverman AP, Kool ET. Quenched autoligation probes allow discrimination of live bacterial species by single nucleotide differences in rRNA. *Nucleic Acids Res.* 33(15), 4978–4986 (2005).

45 Yilmaz S, Haroon MF, Rabkin BA, Tyson GW, Hugenholtz P. Fixation-free fluorescence *in situ* hybridization for targeted enrichment of microbial populations. *ISME J.* 4(10), 1352–1356 (2010).

46 Okabe S, Satoh H, Kindaichi T. A polyphasic approach to study ecophysiology of complex multispecies nitrifying biofilms. *Methods Enzymol.* 496, 163–184 (2011).

47 Behrens S, Losekann T, Pett-Ridge J *et al.* Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence *in situ* hybridization (EL-FISH) and NanoSIMS. *Appl. Environ. Microbiol.* 74(10), 3143–3150 (2008).

•• **Use of nanometer-scale secondary-ion mass spectrometry and FISH to link metabolic activity and species identification in a complex biofilm.**

48 Orphan VJ, House CH, Hinrichs KU, Mckeegan KD, Delong EF. Methane-consuming archaea revealed by directly coupled isotopic and phylogenetic analysis. *Science* 293(5529), 484–487 (2001).

49 Berry D, Mader E, Lee TK *et al.* Tracking heavy water (D2O) incorporation for identifying and sorting active microbial cells. *Proc. Natl Acad. Sci. USA* 112(2), E194–E203 (2015).

50 Hatzenpichler R, Scheller S, Tavormina PL, Babin BM, Tirrell DA, Orphan VJ. *In situ* visualization of newly synthesized proteins in environmental microbes using amino acid tagging and click chemistry. *Environ. Microbiol.* 16(8), 2568–2590 (2014).

- **Description of bio-orthogonal non-canonical amino acid tagging that can be combined with FISH to link taxonomy and translational activity.**

51 Pernthaler A, Amann R. Simultaneous fluorescence *in situ* hybridization of mRNA and rRNA in environmental bacteria. *Appl. Environ. Microbiol.* 70(9), 5426–5433 (2004).

52 Wendeberg A, Zielinski FU, Borowski C, Dubilier N. Expression patterns of mRNAs for methanotrophy and thiotrophy in symbionts of the hydrothermal vent mussel *Bathymodiolus puteoserpentis*. *ISME J.* 6(1), 104–112 (2012).

53 Mota CR, So MJ, de los Reyes FL 3rd. Identification of nitrite-reducing bacteria using sequential mRNA fluorescence *in situ* hybridization and fluorescence-assisted cell sorting. *Microb. Ecol.* 64(1), 256–267 (2012).

54 Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS, Molin S. Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bacteriol.* 182(22), 6482–6489 (2000).

55 Yang L, Liu Y, Markussen T, Hoiby N, Tolker-Nielsen T, Molin S. Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol. Med. Microbiol.* 62(3), 339–347 (2011).

56 Wouters K, Maes E, Spitz JA *et al.* A non-invasive fluorescent staining procedure allows confocal laser scanning microscopy based imaging of *Mycobacterium* in multispecies biofilms colonizing and degrading polycyclic aromatic hydrocarbons. *J. Microbiol. Methods* 83(3), 317–325 (2010).

57 Beaufort S, Da Silva T, Lafforgue C, Alfenore S. Fluorescent proteins as *in vivo* and *in situ* reporters to study the development of a *Saccharomyces cerevisiae* yeast biofilm and its invasion by the bacteria *Escherichia coli*. *FEMS Microbiol. Ecol.* 80(2), 342–351 (2012).

58 Van Gestel J, Weissing FJ, Kuipers OP, Kovacs AT. Density of founder cells affects spatial pattern formation and cooperation in Bacillus subtilis biofilms. *ISME J.* 8(10), 2069–2079 (2014).

59 Bridier A, Briandet R, Bouchez T, Jabot F. A model-based approach to detect interspecific interactions during biofilm development. *Biofouling* 30(7), 761–771 (2014).

- **Using real-time confocal laser scanning microscopy to analyze biofilms formed with fluorescent proteins reporter it was possible to establish a model for the detection of interspecific interactions during biofilm development.**

60 Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat. Methods* 2(12), 905–909 (2005).

61 Mergulhão F, Monteiro GA, Cabral JMS, Taipa MA. Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. *J. Microb. Biotechnol.* 14(1), 1–14 (2004).

62 Teodosio JS, Simoes M, Mergulhao FJ. The influence of nonconjugative *Escherichia coli* plasmids on biofilm formation and resistance. *J. Appl. Microbiol.* 113(2), 373–382 (2012).

63 Zhang T, Pabst B, Klapper I, Stewart PS. General theory for integrated analysis of growth, gene, and protein expression in biofilms. *PLoS ONE* 8(12), e83626 (2013).

64 Hansen MC, Palmer RJ Jr, Udsen C, White DC, Molin S. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 147(Pt 5), 1383–1391 (2001).

65 Patterson GH, Knobel SM, Sharif WD, Kain SR, Piston DW. Use of the green fluorescent protein and its mutants in quantitative fluorescence microscopy. *Biophys. J.* 73(5), 2782–2790 (1997).

66 Habimana O, Meyrand M, Meylheuc T, Kulakauskas S, Briandet R. Genetic features of resident biofilms determine attachment of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 75(24), 7814–7821 (2009).

67 Malone CL, Boles BR, Lauderdale KJ, Thoendel M, Kavanaugh JS, Horswill AR. Fluorescent reporters for *Staphylococcus aureus*. *J. Microbiol. Methods* 77(3), 251–260 (2009).

68 Billi D. Plasmid stability in dried cells of the desert cyanobacterium *Chroococcidiopsis* and its potential for GFP imaging of survivors on Earth and in space. *Orig. Life Evol. Biosph.* 42(2–3), 235–245 (2012).

69 Brzoska AJ, Firth N. Two-plasmid vector system for independently controlled expression of green and red fluorescent fusion proteins in *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 79(9), 3133–3136 (2013).

70 Hoiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O. Antibiotic resistance of bacterial biofilms. *Int. J. Antimicrob. Agents* 35(4), 322–332 (2010).

71 Bridier A, Sanchez-Vizuete P, Guilbaud M, Piard JC, Naitali M, Briandet R. Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* 45(Pt B), 167–178 (2015).

72 Lenz AP, Williamson KS, Pitts B, Stewart PS, Franklin MJ. Localized gene expression in

Pseudomonas aeruginosa biofilms. *Appl. Environ. Microbiol.* 74(14), 4463–4471 (2008).

73 Matsushima R, Watanabe R, Tsuda M, Suzuki T. Analysis of extracellular alginate lyase (alyA) expression and its regulatory region in a marine bacterial strain, *Pseudoalteromonas atlantica* AR06, using a *gfp* gene reporter system. *Mar. Biotechnol. (NY)* 15(3), 349–356 (2013).

74 Garmyn D, Gal L, Briandet R *et al.* Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. *Appl. Environ. Microbiol.* 77(17), 6286–6289 (2011).

75 Rieu A, Briandet R, Habimana O, Garmyn D, Guzzo J, Piveteau P. *Listeria monocytogenes* EGD-e biofilms: no mushrooms but a network of knitted chains. *Appl. Environ. Microbiol.* 74(14), 4491–4497 (2008).

76 Vlamakis H, Aguilar C, Losick R, Kolter R. Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Devel.* 22(7), 945–953 (2008).

77 Lopez D, Vlamakis H, Kolter R. Generation of multiple cell types in *Bacillus subtilis*. *FEMS Microbiol. Rev.* 33(1), 152–163 (2009).

78 Hentschel E, Will C, Mustafi N, Burkovski A, Rehm N, Frunzke J. Destabilized eYFP variants for dynamic gene expression studies in *Corynebacterium glutamicum*. *Microb. Biotechnol.* 6(2), 196–201 (2013).

79 Wessel AK, Hmelo L, Parsek MR, Whiteley M. Going local: technologies for exploring bacterial microenvironments. *Nat. Rev. Microbiol.* 11(5), 337–348 (2013).

80 Schramm A, Larsen LH, Revsbech NP, Ramsing NB, Amann R, Schleifer KH. Structure and function of a nitrifying biofilm as determined by *in situ* hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* 62(12), 4641–4647 (1996).

81 Hidalgo G, Burns A, Herz E *et al.* Functional tomographic fluorescence imaging of pH microenvironments in microbial biofilms by use of silica nanoparticle sensors. *Appl. Environ. Microbiol.* 75(23), 7426–7435 (2009).

82 Liu X, Ramsey MM, Chen X, Koley D, Whiteley M, Bard AJ. Real-time mapping of a hydrogen peroxide concentration profile across a polymicrobial bacterial biofilm using scanning electrochemical microscopy. *Proc. Natl Acad. Sci. USA* 108(7), 2668–2673 (2011).

83 Koley D, Ramsey MM, Bard AJ, Whiteley M. Discovery of a biofilm electrocline using real-time 3D metabolite analysis. *Proc. Natl Acad. Sci. USA* 108(50), 19996–20001 (2011).

84 Reuben S, Banas K, Banas A, Swarup S. Combination of synchrotron radiation-based Fourier transforms infrared microspectroscopy and confocal laser scanning microscopy to understand spatial heterogeneity in aquatic multispecies biofilms. *Water Res.* 64, 123–133 (2014).

85 Ivleva NP, Wagner M, Horn H, Niessner R, Haisch C. Raman microscopy and surface-enhanced Raman scattering (SERS) for *in situ* analysis of biofilms. *J. Biophotonics* 3(8–9), 548–556 (2010).

86 Milucka J, Ferdelman TG, Polerecky L *et al.* Zero-valent sulphur is a key intermediate in marine methane oxidation. *Nature* 491(7425), 541–546 (2012).

87 Kaltenpoth M, Strupat K, Svatos A. Linking metabolite production to taxonomic identity in environmental samples by (MA) LDI-FISH. *ISME J.* 10(2), 527–531 (2016).

88 Daims H, Lucker S, Wagner M. Daime, a novel image analysis program for microbial ecology and biofilm research. *Environ. Microbiol.* 8(2), 200–213 (2006).

89 Schillinger C, Petrich A, Lux R *et al.* Co-localized or randomly distributed? Pair cross correlation of *in vivo* grown subgingival biofilm bacteria quantified by digital image analysis. *PLoS ONE* 7(5), e37583 (2012).

90 Fontenete S, Leite M, Cappoen D *et al.* Fluorescence *in vivo* hybridization (FIVH) for detection of *Helicobacter pylori* infection in a C57BL/6 mouse model. *PloS ONE* 11(2), e0148353 (2016).

91 Knierim B, Luef B, Wilmes P *et al.* Correlative microscopy for phylogenetic and ultrastructural characterization of microbial communities. *Environ. Microbiol. Rep.* 4(1), 36–41 (2012).

- **Association of species identification and subcellular localization of gene expression patterns.**