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A SHORTENED RNA-FISH PROTOCOL FOR ANALYZING ARTWORKS' MICROCOLONIZERS

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ABSTRACT

Microorganisms are involved in the deterioration of Cultural Heritage. Thus, for facilitating the formulation of proper Safeguard strategies there is a need to enhance the techniques used for their detection and identification.

RNA Fluorescent *In Situ* Hybridization (RNA-FISH) has been successfully applied for phylogenetic identification of the viable components of the microbial communities colonizing artworks both *in situ* and *ex situ*. Until recently, it was time-consuming, taking not less than 6 h for the analysis. We have developed an RNA-FISH in suspension protocol that allowed *ex situ* analysis of microorganisms involved in artworks' biodeterioration in 5 h. In this work, three modified protocols, involving microwave heating, were evaluated for further shortening two of the four main critical steps in RNA-FISH: hybridization and washing.

The original and modified protocols were applied in cellular suspensions of bacteria and yeast isolates. The results obtained were evaluated and compared in terms of detectability and specificity of the signals detected by epifluorescence microscopy.

One of the methods tested showed good and specific FISH signals for all the microorganisms selected and did not produce signals evidencing non-specific or fixation-induced fluorescence.

This 3 h protocol allows a remarkable reduction of the time usually required for performing RNA-FISH analysis in Cultural Heritage samples. Thus, a rapid alternative for analyzing yeast and bacteria cells colonizing artworks' surfaces by RNA-FISH is presented in this work.

INTRODUCTION

Artworks and tangible Cultural Heritage are an invaluable legacy that need to be preserved. Multiple factors contribute to their deterioration (pH, temperature and humidity among others) [1,2]. Microorganisms (bacteria, yeast, filamentous fungi, lichens and algae) are key-players in this process [3]. In fact, the microbial colonization and subsequent deterioration of artworks and assets belonging to our Cultural Heritage are well documented phenomena [2–5].

In this way, it is crucial to detect the microbial communities thriving in Cultural Heritage materials, and particularly those metabolically active (potential biodeteriogenic microorganisms), in order to formulate appropriate strategies for the conservation and safeguard of Cultural Heritage [2–5]. Consequently, enhancement of the techniques used for microbiological analysis is required [1].

RNA Fluorescence *In Situ* Hybridization (RNA-FISH) is a phylogenetic staining technique that allows to detect and identify the metabolically active components of the microbial community present in a sample [6]. It has been successfully applied in complex matrix: blood, soils, food and cultural heritage surfaces *inter alia* [7–15].

Although protocols for FISH might differ significantly, the general methodical procedure involves: i) fixation/ permeabilization of the cells for allowing the entry of the fluorescent probes; ii) hybridization of the probe to the target sequence into the cell; iii) removal of unbound and excess probes by washing; and, iv) analysis of the cells by microscopy or flow cytometry [6]. Various RNA-FISH protocols have been already described for analyzing the potential biodeteriogenic microbial colonizing cultural objects and assets in tape-strips samples or microsamples [11,16–18]. These protocols, are time-consunimg taking more than 6 h.

An in-suspension RNA-FISH protocol for *ex situ* analysis of the microbial community in 5 h has been recently reported by us [14]. The aim of this work is to enhance this protocol to further reduce the time required for the analysis of the samples. With this purpose, various hybridization methods have been tested in combination with a reduced washing step in an attempt to shorten hybridization and washing times.

Microwaving of the cells were considered as part of the hybridization and/or washing steps of the alternative protocols proposed in this work for saving time in the analysis of Cultural Heritage samples. It has been proved to be a controllable way of accelerating most processes involving diffusion and many chemical reactions [19]. It is chiefly applied to stimulate fixation and reduce decalcification, staining and immunostaining times [19]. Furthermore, microwave heating has been successfully applied before to improve and shorten various steps of the RNA-FISH analysis enhancing the hybridization signals in clinical and food samples [20–24].

Thus, the results obtained using the original and modified protocols were evaluated and compared in terms of detectability and specificity of the signals detected by epifluorescence microscopy.

MATERIALS AND METHODS

Strains and growth conditions

The microorganism used in this work were two yeast (*Rhodotorula* sp. *and Saccharomyces cerevisiae* CCMI 396*)* and three bacteria strains (*Escherichia coli* ATCC 25922 and *Arthrobacter* sp. 1 and Arthrobacter sp. 2). Two of them are reference strains: *Saccharomyces cerevisiae* and *Escherichia coli*. They were obtained from culture collections (CCMI - Culture Collection of Industrial Microorganisms- and ATCC -American Type Culture Collection- de Manassas). The other microorganisms were isolated from artworks' samples and belong to the HERCULES-Biotech Lab collection, Évora University.

Bacteria and yeast strains were stored at 4°C in Nutrient Agar (NA) and Yeast Extract Peptone Dextrose Agar (Yeast Agar) slants, respectively, and maintained by periodic transferring.

Liquid cultures were prepared by adding 1 ml inoculum in 100 ml of Nutrient broth (for bacteria growth) or Yeast Extract Peptone Dextrose Broth (for yeast growth) to a 250 mL sterile Erlenmeyer flask. They were incubated at 30ºC with orbital shaking at 120 rpm and the cells were harvested in the exponential phase of growth.

RNA-FISH

Cellular suspensions of isolates were used for simulating the cellular suspensions resulting from the recovery of the cells from the Cultural Heritage samples in suspension [25].

RNA-FISH was performed with slight modification of the standard FISH protocol previously described by us for detecting microorganisms involved in Cultural Heritage biodeterioration [25]. Four protocols were applied using various hybridization and washing conditions (Table 1).

Table 1. Conditions of the four main RNA-FISH steps in the protocols evaluated in this study.

	Fixation / Permeabilization	Hybridization	Washing	Analysis
P1	Absolute EtOH 1 h room temperature	15 s microwaving 15 min incubation 15 s microwaving 15 min incubation	10 s microwaving 7.5 min incubation	Epiflluorescence microscopy
P2		30 s microwaving 30 min incubation		
P3		30 min incubation		
P4		2 h incubation	30 min incubation	

For microwaving, the microtubes were placed in the center of the rotating plate and were directly and individually irradiated using a domestic microwave oven (KUNFT 17MX02) with a maximal nominal output power of 700 W and a frequency of 2450MHz. The microwave was adequately calibrated to adjust the outsource power to 200W.

All the incubations were carried out in a water bath at 46ºC.

Four different assays were carried out for evaluating each RNA-FISH protocol by varying the probes added in the hybridation step: i) two conventional assays were performed by adding fluorescently labeled complementar probes to 16S/18S rRNA regions of bacteria and yeast, respectively. Universal FISH probes labeled with Cy3 or 6-FAM at the 5'end were used (EUK516-CY3 and EUK516-6-FAM for yeast and EUB338-Cy3 and EUB338-6-FAM for bacteria); ii) a control for fixation-induced fluorescence was performed without probe addition to the hybridization suspension; iii) a negative control for hybridization specificity was performed by the addition of a non-complementar probe to the rRNA of the target microorganism (EUK516-Cy3 non-complementar to bacteria and EUB338-Cy3 non-complementar to yeast).

Oligonucleotide probes

Universal oligonucleotide probes EUB338 (5'-TGCTGC CTCCCGTAGGAGT-3') and EUK516 (5'- ACC AGA CTT GCC CTC C -3') labelled at the 5'end with Cy3 or 6-FAM were used [26,27]. They targeted to independent sites of the 16S/18S rRNA molecule of microorganisms belonging to the domains Bacteria and Eukarya, respectively. The probes were supplied by NZYTech, genes & enzymes (Lisboa, Portugal).

Microscopic analysis

A biological microscope BA410E Motic equipped with a 100W Quartz Halogen Koehler illumination with intensity control and with an epi-attachment (EF-UPR-III) and a Power Supply Unit (MOTIC MXH-100). Microphotographs were

acquired using a MoticamPRO 282B camera coupled to the microscope and to visualize and analyze them the Motic Images Plus 2.0LM software was used.

Filter TRITC (TRITC (Rhodamine)/DII/Cy3 set, Motic: excitation D540/25x, dichroic mirror 565DCLP, and emission D605/55m) and FITC (FITC/RSGFP/Fluo 3/DiO Acridine Orange [+RNA] set, Motic: excitation D480/30x, dichroic mirror 505DCLP and emission D535/40m) were used for microscopic inspection of the samples mounted in microscope slides.

RESULTS AND DISCUSSION

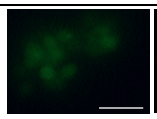
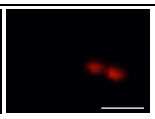
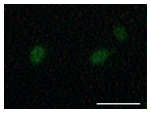
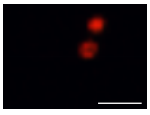
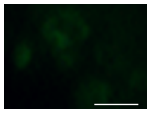
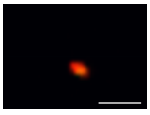
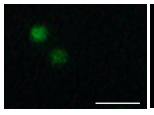
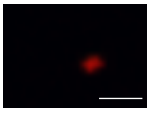
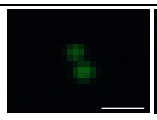
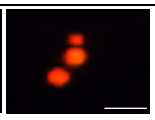
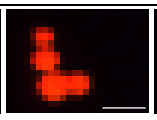
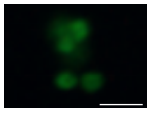
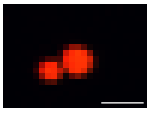
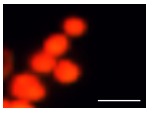
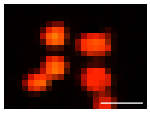
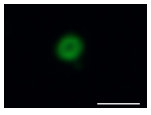
In an attempt to reduce the time necessary for analyzing the microorganisms involved in the biodeterioration of Cultural Heritage by RNA-FISH in suspension various time-saving alternatives were evaluated in this work. The protocol considered as starting point has a duration of 5 h and the alternative protocols tested [25], with shorter hybridization and washing steps (Table 1), could allow to save more than 1 h and 50 min.

The microphotographs obtained by epifluorescence microscopy using the four different protocols (P1-P4) for the yeast and bacteria cells investigated are shown in Tables 2 and 3, respectively.

None of the microorganisms investigated showed fluorescence before the RNA-FISH application (data not shown) and neither after performing the RNA-FISH assay without addition of probe (fixation-induced fluorescence control, Tables 2 and 3). This indicated that, independently of the cell treatment applied after fixation the fixative used (absolute ethanol) did not react with cellular components inducing fluorescence. This is one of the advantages of the protocols presented here over other RNA-FISH methods applied for analyzing Cultural Heritage samples. They commonly involved paraformaldehyde fixation which is associated to fixation-induced fluorescence (which can contribute to background fluorescence hampering the detection of the FISH signals)[10,13,28]. Also, previous investigations on clinical applications of RNA-FISH have allowed to concluded that background noise is reduced to the minimum by the use of microwave-assisted protocols [29].

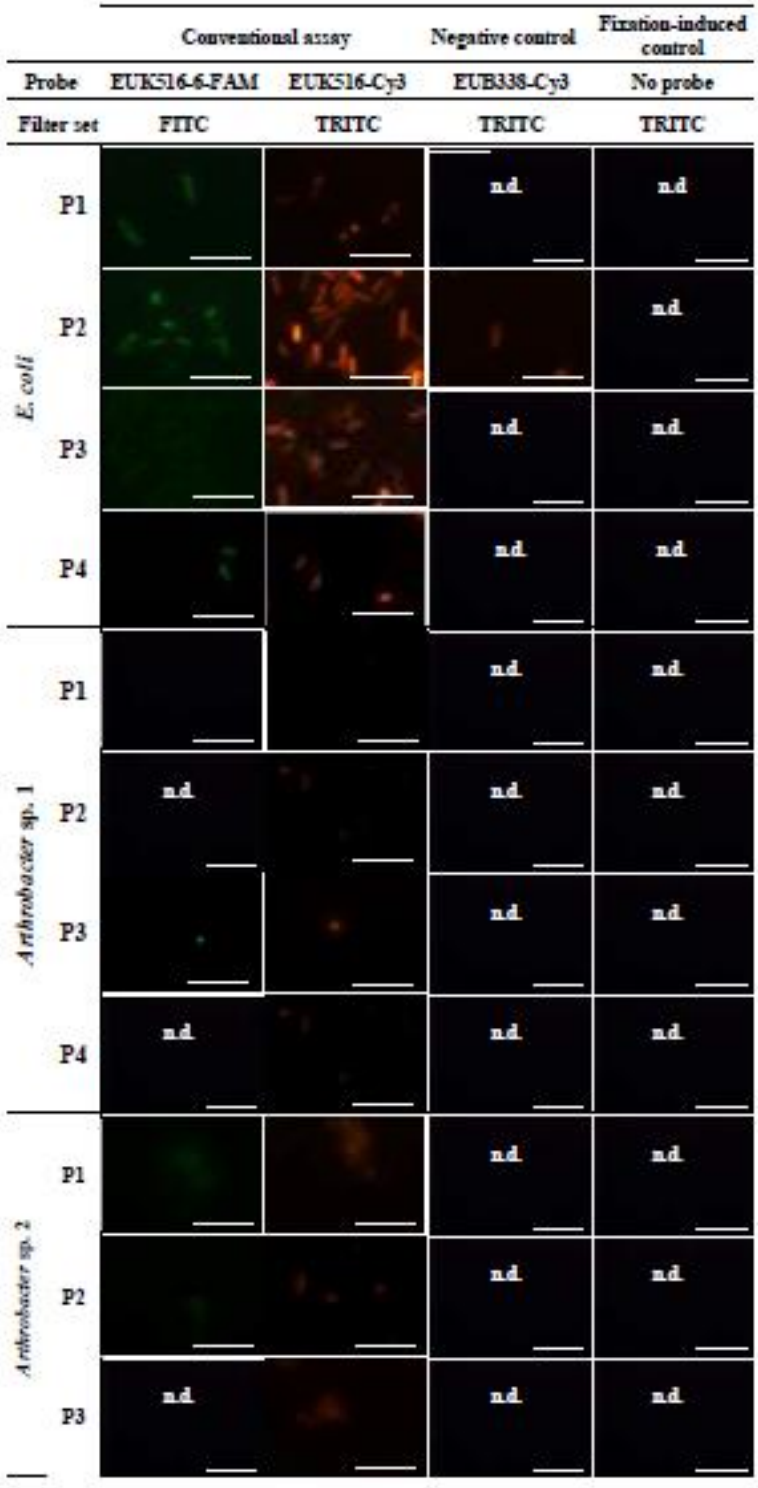
It is noteworthy to mention that all the methods tested preserved the cell morphology and yielded intense and stable fluorescent signals with the Cy3 labeled probes specific for the target microorganisms (Tables 2 and 3). However, weak or even non-detectable signals were observed for the bacteria stained with EUB338-6-FAM. Also, although yeast cells stained with EUK516-6-FAM exhibited intense fluorescence during microscopic inspection of the cells, the 6-FAM fluorescence intensity decayed rapidly and almost disappeared after 45 s of irradiation (Figure 1), particularly in the samples treated with microwaves. Thus, for avoiding false negatives using this RNA-FISH protocols: i) observations under the microscope need to be performed minimizing the exposition of the samples to the excitation light; and/or ii) dyes with high photostability should be used for labeling the probes.

Table 2. Microphotographs of yeast cells captured by epifluorescence microscopy after the application of the four different RNA-FISH protocols. Scale bar represents 10 µm and non-detected signals are indicated by n.d.

		Conventional assay		Negative control	Fixation-induced control
	Probe	**EUK516-6-FAM**	**EUK516-Cy3**	**EUB338-Cy3**	**No probe**
	Filter set	**FITC**	**TRITC**	**TRITC**	**TRITC**
***Rhototorula* sp.**	**P1**			n.d.	n.d.
	P2			n.d.	n.d.
	P3			n.d.	n.d.
	P4			n.d.	n.d.
S. cerevisiae	**P1**				
	P2				n.d.
	P3			n.d.	n.d.
	P4			n.d.	n.d.

Whereas, according to the results analyzed until now all the modified protocols seemed to be potential time-saving alternatives to the original one, they were not. The microphotographs captured for the negative controls (Tables 2 and 3) revealed that for most of the microorganisms investigated (*Rhodotorula* sp. and *Arthrobacter* sp.) the hybridization/washing conditions tested have adequate stringencies to avoid the apparition of non-specific fluorescent signals. However, non-specific fluorescence was observed when microwaves were used in the hybridization step (protocols P1 and P2) for *S. cerevisiae* and *E. coli*. These results discarded the possibility of using P1 and P2 protocols for detecting and identifying the target cells, as they could lead to false positives.

Table 3. Microphotographs of bacteria cells captured by epifluorescence microscopy, after the application of the four different RNA-FISH protocols. Scale bar represents 10 µm and non-detected signals are indicated by n.d.



However, the samples treated with the P3 and the original protocol (P4) yielded intense specific fluorescence and did not show non-specific fluorescence for all the microorganisms investigated in this study. Thus, the P3 method revealed to be a potential time-saving alternative to the P4 method that allowed to reduce the time required to analyze the Cultural Heritage samples to the third part, from 5h to about 3h.

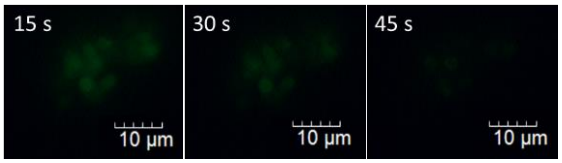


Figure 1. Decay of EUK516-6-FAM fluorescence along time of exposure to excitation light. Epifluorescence microphotographs corresponding to *Rhodotorula* sp. cells stained with EUK516-6-FAM using the P1 protocol and observed under the FITC filter set during 15, 30 and 45 s.

Thus, the results pointed out the possibility of shortening the original RNA-FISH protocol for *ex situ* analysis of artworks' microcolonizers by reducing the hybridization and washing times.

CONCLUSION

A time-saving alternative for analyzing bacteria and cells colonizing artworks' surfaces *ex situ* by RNA-FISH was developed, reducing the time required for the analysis from 5 to 3 h.

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NOMENCLATURE

RNA-FISH (RNA Fluorescence *In Situ* Hybridization)

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