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Gold-FISH: A new approach for the *in situ* detection of single microbial cells combining fluorescence and scanning electron microscopy

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ABSTRACT

A novel fluorescence *in situ* hybridisation (FISH) method is presented that allows the combination of epifluorescence and scanning electron microscopy (SEM) to identify single microbial cells. First, the rRNA of whole cells is hybridised with horseradish peroxidase-labelled oligonucleotide probes and this is followed by catalysed reporter deposition (CARD) of biotinylated tyramides. This facilitates an amplification of binding sites for streptavidin conjugates covalently labelled with both fluorophores and nanogold particles. The deposition of Alexa Fluor 488 fluoro-nanogold–streptavidin conjugates was confirmed *via* epifluorescence microscopy and cells could be quantified in a similar way to standard CARD–FISH approaches. To detect cells by SEM, an autometallographic enhancement of the nanogold particles was essential, and allowed the *in situ* localisation of the target organisms at resolutions beyond light microscopy. Energy dispersive X-ray spectroscopy (EDS) was used to verify the effects of CARD and autometallography on gold deposition in target cells.

The gold-FISH protocol was developed and optimised using pure cultures and environmental samples, such as rice roots and marine sediments. The combination of epifluorescence and scanning electron microscopy provides a promising tool for investigating microorganisms at levels of high resolution. Correlative characterisation of physicochemical properties by EDS will allow for the analysis of microbe-surface interactions.

Introduction

Fluorescence *in situ* hybridisation (FISH) is routinely used for the phylogenetic identification and enumeration of microorganisms on a single-cell level in environmental studies [4]. Fluorescently labelled oligonucleotide probes are hybridised to highly conserved regions of microbial rRNA and detected by epifluorescence or confocal laser scanning microscopy [1,6]. However, to date, the detection of the resulting signals has been restricted by the resolution and the sensitivity of fluorescence microscopy. For increased sensitivity, FISH combined with catalysed reporter deposition (CARD–FISH) was introduced into molecular ecology [24]. The application of CARD–FISH to environmental samples was shown to amplify fluorescent signals in comparison to monolabelled probes and, thus, to increase significantly the detection rates of microorganisms [8,11,16].

To investigate interactions of microbial communities in soil, biofilms, flocs, and slimes on the micro- and nano-scale, sophisticated methods allow the physicochemical characterisation of microbial habitats [18,36]. The study of material or plant surfaces would benefit especially from complementary identification and visualisation of microorganisms at higher levels of resolution [5]. X-ray based techniques, such as spectroscopy or micro computed tomography (μCT), have become increasingly popular in environmental studies and rely on the detection of elemental characteristics. Labelling cells with elements of high density is therefore desirable for the phylogenetic identification of single microbial cells on the same level as that commonly achieved for rRNA using FISH.

Deposition of gold and silver particles is frequently used for immunolabelling of specimens analysed by electron microscopy in histochemical studies [13,28]. For a whole-cell identification of microorganisms at a high resolution level, *in situ* hybridisations (ISH) have recently been combined with scanning electron microscopy (SEM) [7,12,17]. Other studies have investigated microbial ultrastructures by applying ISH and transmission electron microscopy (TEM), which have allowed for a detailed analysis of intracellular compounds such as magnetosomes [30,35]. All of these studies have used either oligonucleotide probes directly labelled with nanogold or nanogold-labelled antibodies that targeted probes with fluorescein or digoxigenin. A major drawback is that successful hybridisation and nanogold deposition can only be

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verified by electron microscopy. Thus, a rapid pre-screening for the successful deposition of the nanogold label, as well as the quantification of single cells detected by fluorescence microscopy, is not possible. Furthermore, the sensitivity of these approaches is limited by the number of metal particles deposited, which is linear to the number of ribosomes per cell. In this respect, these methods resemble FISH with monolabelled probes, which often show weak signal intensities and low signal-to-noise ratios in environmental samples [3,32]. To improve the sensitivity of immunogold detection, the amplification of binding sites for gold-labelled antibodies has been used in histochemical studies [21,25,27,31].

To date, there is no technique available that combines: (i) the phylogenetic identification and enumeration of individual microorganisms by epifluorescence microscopy, with (ii) the identification and *in situ* localisation of nanogold-labelled target cells on an ultrastructural level by SEM. In this study, "gold-FISH" is presented, which is a novel protocol where both fluorophores and nanogold particles are deposited in bacterial cells after a single hybridisation event. The protocol uses HRP catalysed reporter deposition (CARD) of biotinylated tyramides that, in a subsequent reaction, are bound by streptavidin conjugated to the Alexa Fluor 488 fluorophore and a nanogold particle. Such a double-labelling of cells is useful for reliable development and application of the new protocol compared to CARD–FISH. In samples containing mixed microbial communities, a rapid pre-screening *via* fluorescence microscopy is especially advantageous prior to SEM analyses at resolutions beyond light microscopy. In combination with techniques describing physicochemical conditions, gold-FISH has the potential to provide new insights into microbe-habitat interactions in microbial ecology.

Materials and methods

Preparation of samples

Pure cultures of *Escherichia coli* (JM107, Fermentas), *Bacillus subtilis* (DSMZ), a *Roseobacter*-related strain AK199 and mixed cultures thereof were used to optimise the gold-FISH protocol. Samples of a marine tidal sediment from the German North Sea coast [19], a Chinese paddy soil [26], and roots of sterile-grown wetland rice (*Oryza sativa* L.) inoculated with *Rhizobium leguminosarum* were selected as environmental samples. All samples were fixed in 4% [v/v] formaldehyde solution in 1× phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) for 2.5 h at 4 °C. After two washing steps in 1× PBS, the samples were stored in 1× PBS/ethanol (1:1) at −20 °C. The fixed soil and sediment samples were sonicated and filtered on white polycarbonate filter membranes (0.2 μm pore size; Millipore). Filter membranes were embedded in 0.2% low melting point agarose and dehydrated in ethanol. For cell wall permeabilisation, the filters were consecutively incubated with lysozyme solution (10 mg mL^{-1}; 60 min) and achromopeptidase solution (60 U mL^{-1}; 30 min) at 37 °C. Endogenous peroxidases were inactivated by incubation of filters in methanol containing 0.15% hydrogen peroxide (H_2O_2) for 30 min at room temperature (RT). Unspecific binding of streptavidin conjugates to endogenous biotin present in cells and environmental samples was prevented by a pre-treatment with a streptavidin/biotin blocking kit (SP-2002; Vector Laboratories).

In situ hybridisation

In situ hybridisation was performed with oligonucleotide probes targeting: (i) the 16S rRNA in the domain *Bacteria* (EUB338; 5′-GCT GCC TCC CGT AGG AGT-3′) [2], (ii) the 23S rRNA in the gamma subclass of *Proteobacteria* (GAM42a: 5′-GCC TTC CCA CAT CGT TT-3′) [20], and (iii) the 16S rRNA of marine *Roseobacter*-clade bacteria (ROS537; 5′-CAA CGC TAA CCC CCT CC-3′) [10]. Probe NONEUB (5′-ACT CCT ACG GGA GGC AGC-3′) [33] served as a negative control. Probes were labelled with horseradish peroxidase (HRP) or biotin at the 5′-end (Biomers, Ulm, Germany).

Filter sections were incubated in 400 μL hybridisation buffer (10% dextran sulphate (w/v), 0.9 M NaCl, 20 mM Tris–HCl, 35% formamide, 10% blocking reagent (Roche), 0.01% SDS) containing 1.5 μL probe working solution (50 ng μL^{-1}) for at least 2 h at 35 °C while rotating. Subsequently, filter sections were washed in prewarmed washing buffer (42 mM NaCl, 20 mM Tris–HCl, 5 mM EDTA, 0.01% SDS; 5 min at 37 °C), H_2O_{MQ} (2 min at RT), and PBS–Triton-X100 (0.05% Triton-X100, Bio-Rad in 1× PBS; 15 min at RT).

To compare the efficiency of different FISH approaches, a biotin-labelled probe (EUB338) analogous to FISH using monolabelled oligonucleotide probes was also used. In the following, it is referred to as "mono-gold-FISH". In this approach, final probe concentrations of 2.5 and 5.0 ng μL^{-1} were used for hybridisations at 46 °C (4 h). Washing was consecutively performed in prewarmed washing buffer (70 mM NaCl, 20 mM Tris–HCl, 5 mM EDTA, 0.01% SDS; 5 min at 48 °C), H_2O_{MQ} (2 min at RT), and PBS–Triton-X100 (15 min at RT). Afterwards, filter sections were incubated directly with the streptavidin conjugate.

Catalysed reporter deposition (CARD)

For catalysed reporter deposition (CARD) with biotinylated tyramides, the filter sections hybridised with HRP-labelled probes were incubated in 500 μL of freshly prepared amplification buffer (10% dextran sulphate [w/v], 2 M NaCl, 1% [v/v] blocking reagent in 1× PBS) amended with 0.0015% H_2O_2 [v/v] and 0.004% [v/v] biotinylated tyramide solution (20 mg mL^{-1} dimethylformamide containing 2% [w/v] 3-iodophenolboronic acid) for 20 min at 37 °C while rotating. Afterwards, the filter sections were consecutively washed in 1× PBS–gelatine–Tween-20 (PGT; 0.1% gelatine [w/v] (Fluka), 0.1% Tween-20 (Sigma–Aldrich); 10 min at RT), and H_2O_{MQ} for 10 min at RT while rotating.

For standard CARD–FISH, a fluorescein-labelled tyramide solution was added to the amplification buffer and incubated as described above. The filter sections were then each washed in 1× PBS–Triton-X100 and in H_2O_{MQ} for 10 min at RT, and subsequently mounted on glass slides for epifluorescence microscopy.

High affinity binding of fluoro-nanogold–streptavidin conjugates

Filter sections used for CARD with biotinylated tyramides were incubated in 400 μL 1× PBS–BSA (1% albumin fraction V, Roth) containing 0.25% of a streptavidin conjugate covalently labelled with a 1.4 nm nanogold particle and 2–3 Alexa Fluor 488 (AF 488) fluorophores (0.08 mg mL^{-1}, Alexa Fluor® 488 FluoroNanogold™-Streptavidin, Nanoprobes) for at least 2 h at 35 °C while rotating. The filter sections were then washed in PGT (twice for 10 min at RT) and H_2O_{MQ} for 10 min at RT while rotating.

Epifluorescence microscopy and cell enumeration

For the evaluation of fluorescent signals, air-dried filter sections were covered with VectaShield H-1200 medium containing 1.5 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories), mounted on glass slides, and covered with a cover slip. Slides were evaluated by epifluorescence microscopy using a ZEISS Axioskop 2 microscope equipped with a HBO 100 W mercury vapour lamp and a ZEISS Neofluar objective (63×; Carl Zeiss). An ocular with an integrated counting grid (10 × 10 1.25 mm squares; Carl Zeiss) was used for cell enumeration. The cells in 20 squares were counted at 15 randomly selected microscopic fields for each filter. Fluorescein and Alexa Fluor 488 stained cells were