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New Periodontal Pathogens and their Biogeography in *ex Vivo* Biofilms

Fany B. Ocampo

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Abstract

Background: About 35% of the oral microbiome remains uncultured due to limitations of conventional laboratory techniques. More than 200 of those phylotypes have been catalogued in the HOMD and a subset of them had been proposed as candidate periodontal pathogens. This segment of the microbiome merits further investigation, as it might harbor important pathogens that are currently overlooked. **Objective:** The objective of this study was to devise an imaging approach to study such phylotypes in the conditions most conducive to their growth and begin to unveil their ecological and biogeographical characteristics.

Methods: Previous work from the Teles Lab had identified the most common candidate periodontal pathogenic phylotypes and developed biofilms that fostered their growth. Such biofilms and their spent media were used for the development of the imaging approach. 16S rRNA sequencing data from 18 ex vivo biofilms developed from samples collected from 16 periodontitis patients were screened to determine the most common phylotypes. Given the unculturability of phylotypes, pure and mixed cultures of reference strains (*Actinomyces israelii*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*) were used to develop the method. Specific and eubacterial probes targeting 16S rRNA of the taxa of interest were synthesized and tested on pure and mixed cultures and on ex vivo biofilm samples. Fixation and permeabilization protocols were tested and optimized. Biofilm and media samples were visualized using confocal (Leica SP8 and Zeiss LSM 880) and epifluorescence (Leica DM6000B) microscopes.

Results: The microbial screening of 1311 samples from 16 periodontitis patients showed that *Megasphaera* HOT 123, *Prevotella* HOT 526, *Prevotella* HOT 315, *Aggregatibacter* HOT 898 and *Alloprevotella* HOT 912 were the most prevalent and abundant phylotypes. Imaging of pure and mixed cultures of *A. israelii*, *P. gingivalis* and *F. nucleatum* and the use of positive and negative controls demonstrated the specificity of the probes used. Spent media samples were better visualized than biofilm samples. *P. gingivalis* and *F. nucleatum* could be observed in several samples. *Megasphaera* HOT 123 could be clearly visualized as small cocci in media samples. **Conclusions:** The imaging method devised allowed the specific visualization of phylotype *Megasphaera* HOT 123 as cocci located in clusters within ex vivo biofilm and media samples.

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NEW PERIODONTAL PATHOGENS AND THEIR BIOGEOGRAPHY IN EX VIVO
BIOFILMS

Fany Briseyda Ocampo

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ABSTRACT

NEW PERIODONTAL PATHOGENS AND THEIR BIOGEOGRAPHY IN EX VIVO BIOFILMS

Fany Briseyda Ocampo
Flavia Teles, DDS, MS, DMSc

Background: About 35% of the oral microbiome remains uncultured due to limitations of conventional laboratory techniques. More than 200 of those phylotypes have been catalogued in the HOMD and a subset of them had been proposed as candidate periodontal pathogens. This segment of the microbiome merits further investigation, as it might harbor important pathogens that are currently overlooked. **Objective:** The objective of this study was to devise an imaging approach to study such phylotypes in the conditions most conducive to their growth and begin to unveil their ecological and biogeographical characteristics.

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LIST OF ABBREVIATIONS

BHI	Brain Heart Infusion
CBD	Calgary Biofilm Device
CLASI-FISH	Combinatorial Labeling and Spectral Imaging FISH
Cy	Cyanine
FAM	Fluorescein
FISH	Fluorescence In Situ Hybridization
HA	Hydroxyapatite
HOMD	Human Oral Microbiome Database
HOT	Human Oral Taxon
NAM	N-acetylmuramic acid
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Probing Depth
PRAS	Pre-reduced Anaerobically Sterilized media
T-Soy	Trypticase Soy

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CHAPTER 1: INTRODUCTION

Periodontitis is the result of an inflammatory response to a dysbiotic microbiome in susceptible individuals [41]. Periodontitis affects more than 47% of US adults, leading to inflammation, bone and tooth loss [1], possibly contributing to systemic conditions, including respiratory diseases and heart disease [2].

Periodontal pathogens have been recognized for years [3,4] and have provided guidance in disease diagnosis, treatment and prevention [5]. In a benchmark study, Socransky et al [4] identified five bacterial complexes within the periodontal microbiome. More than 13,000 subgingival plaque samples from 185 patients were assessed for the levels of the 40 most common periodontal bacterial species. Using principal component analyses and community ordination, the authors observed significant clustering of certain species as they related to periodontal health and disease. The authors also observed an ecological relationship of microbial succession amongst the clusters and a close association between the red (*Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) and orange complex. They proposed that the colonization by members of the orange complex preceded the colonization by the red complex species. In addition, in the context of clinical parameters, there was a clear positive correlation between higher prevalence of red and orange complex bacteria and increasing pocket depth [4].

Since then, several studies have relied on the pathogenic and health-compatible complexes proposed by Socransky et al [4] to study periodontitis pathogenesis [3] periodontal disease activity, risk [5] and response to treatment. However, the oral cavity harbors more than 700 taxa [6,7]. About 50% of them are cultivated species, such as the species present in the complexes mentioned above. However, close to 35% of those taxa remain uncultured, limiting our ability to study any pathogenic role they may have in

periodontal pathogenesis. It is likely that at least a subset of these more than 240 taxa are pathogens that have remained overlooked. And it was the technological advances that followed the landmark study by Socransky et al [4] that permitted us to go from the cultivation and characterization of thousands of samples collected from hundreds of patients which was slow and laborious to faster and less costly techniques like checkerboard DNA-DNA hybridization technique. This technique allowed the enumeration of multiple taxa in multiple samples at one time [46]. Later it was the open ended culture independent techniques that gave us the advantage of not having to focus on a predetermined set of bacterial species, as it had been the case for checkerboard DNA-DNA hybridization [4], PCR [42], RT-PCR [43] or in-situ hybridization [44]. The amplification of conserved areas of a ubiquitous bacterial gene (16S rRNA gene) by a highly sensitive method (PCR) [45] allowed the identification of all microbial taxa present in a given sample.

The potential role of the uncultivated segment of the periodontal microbiome was investigated by Kumar et al [8], by Quantitative 16S Clonal Analysis. The results revealed the association of several uncultivated taxa with periodontitis, including, *Megasphaera* oral clones BB166, MCE3_141, and BS073 and *Desulfobulbus* oral clones CH031 and R004. Often times, several of the phylotypes studied outnumbered the classical pathogens. Further evidence for these results was provided in the study by Griffen et al [9] who also found similar prevalence of these uncultivated bacteria. Of interest was that she also noted that *Filifactor alocis*, was just as prevalent as *P. gingivalis* and *T. denticola* in diseased sites [9] which has not been previously associated with periodontitis.

With the advances and cost-effectiveness of sequencing platforms, since then several additional studies demonstrated the presence of uncultured bacterial in periodontal biofilm samples [8,9]. In an effort to determine the current weight of evidence

for the newly identified periodontal pathogens, Perez-Chaparro et al [10] conducted a systematic review of the published studies that employed culture-independent techniques to study the periodontal microbiome. The authors sought studies where taxa were found in statistically significantly higher levels and/or proportion and/or prevalence and/or abundance in periodontitis than in periodontal health. Then results were classified into categories: “moderate evidence” if the taxon was found in 3 to 5 studies or in “some evidence” if the taxon was found in 2 studies. Their results showed moderate evidence in the literature to support the association of periodontitis with 17 phylotypes, including *Desulfobulbus* sp. HOT 041, *Fretibacterium* sp. HOT 360 and *TM7* sp. HOT 356.

Even though sequencing studies have allowed the study of the oral microbiome in much greater depth and breadth, they are only the first step to study new pathogens. They only allow the association of uncultured taxa with a given disease parameter. In order to consider them pathogens, certain factors need to be considered. Typically, they involve the fulfillment of the modified Koch’s postulates [11]. They propose that a pathogenic species is enriched at sites of pathology and the corollary that the organism is in lower numbers or proportions or absent in healthy sites or sites with different forms of disease (association). Also, it should be suppressed in a lesion by mechanical debridement or chemotherapeutic agents (elimination). In addition, it should have virulence factors, elicit an immune response and cause disease in animal models [11]. By the nature of sequencing studies, they can only address the association and elimination postulates. The study of virulence, immune response and animal models can only be achieved with the isolation and cultivation of the phylotype organisms of interest.

The unculturability of those taxa stem from several factors [12]. Certain species might require extended incubation periods, much beyond the 7-10 days typically used for oral species. Inter and intra cellular communication, which might give chemical messages

that influence bacterial growth, are disrupted in typical culture protocols. Certain species, known as microbial weeds, may grow at a much higher rate than the rest of the uncultured taxa, which cannot grow in the subsequently depleted and overpopulated media. Finally, many of the cultivation steps disrupt syntrophic interactions. Such interaction is needed by certain species, which might require substrates from neighboring species for growth (also known as helper species). By addressing those factors, it will be possible to promote the growth of uncultivated candidate pathogens and study their metabolism.

Another approach to learn more about the metabolism of those phylotypes and foster their cultivation is by studying their biogeography [13]. The localization of bacterial cells with a biofilm is not random. Rather, it is a consequence of their ecological relationships with other taxa and the environment. Classical studies using immunohistochemistry [4] demonstrated how red complex bacteria are primarily at the deeper portions of the periodontal pocket, while yellow complex species were localized in the shallower portions. Such organization is in line with the metabolism of those organisms, as red complex species are anaerobic, proteolytic and hemin-dependent. Therefore, the (ulcerated) epithelial lining and accessible inflamed connective tissue and gingival crevicular fluid (GCF) are conducive for their growth. Similarly, the yellow complex is comprised primarily by *Streptococcus*, which are facultative anaerobes and can tolerate oxygen and mostly saccharolytic, hence benefiting from the proximity to saliva.

A more recent study expanded those observations by studying supragingival plaque using CLASI-FISH. Mark-Welch et al [14] described a cauliflower structure, where the perimeter was composed of *Streptococcus* and *Haemophilus/Aggregatibacter* on the distal tips of *Corynebacterium* filaments, what she calls the foundation or anchor of the biofilm. Just below that, in the annulus, there are *Fusobacterium* and *Leptotrichia*. This arrangement tells us that these bacteria are benefitting from each other, what can be

identified as helper species. The bacteria that are facultative anaerobes like *Streptococcus* use the tips of *Corynebacterium* to be near oxygen, sugar and saliva as a nutrient source. While the *Fusobacterium* and *Leptotrichia* are near the streptococci, which create the anaerobic environment, they thrive in

Thus, by imaging uncultured organisms in biofilms samples derived from periodontitis patients it will be possible to make inferences about their ecological interactions, which ultimately can provide valuable insights into their growth requirements.

CHAPTER 2: OBJECTIVE

The objective of this study was to develop a protocol to identify the spatial relationships and distribution of uncultured periodontal microorganisms in periodontitis ex-vivo biofilms through imaging. The central hypothesis is that their organization results from interactions with community members and their habitat, which have not yet been reproduced in vitro. The importance of examining the organization and biogeography of these uncultured bacteria within ex-vivo biofilms that it will provide information about their location within the biofilm and relationships with other bacterial species. Collectively, this knowledge will shed light onto their metabolism, their growth requirements and potential pathogenic role in periodontal diseases.

CHAPTER 3: MATERIALS & METHODS

The method developed in this project benefited from the samples generated in a previous study of the Teles lab. As part of that study, sixteen periodontitis patients were recruited at the University of North Carolina at Chapel Hill School of Dentistry. Inclusion criteria was good general health, at least 20 teeth, age 21-80, at least 4 teeth with probing depth (PD) of >4 mm, clinical attachment level (CAL) of >2 mm, excluding 3rd molars and presence of at least 2 sites with PD of > 6 mm. Subjects were excluded if they had received periodontal or antibiotic therapy in the previous 6 months, had any systemic condition that requires antibiotic coverage for periodontal procedures or if they were smokers. A calibrated dental examiner complete examination including pocket depth (PD), clinical attachment level (CAL) and bleeding on probing (BOP) in 6 sites per tooth.

Four subgingival biofilm plaque samples were collected per subject. Prior to sampling, the area was isolated from saliva using cotton rolls and supragingival plaque was removed. The samples were collected from the 4 deepest qualifying sites using sterile Gracey curettes. Samples were then all pooled by placing them all in one tube containing 9 ml of pre-reduced anaerobically sterilized (PRAS) media.

The Calgary Biofilm device (CBD) was used to develop the biofilm model. It consists of a 96 well plate where the lid has 96 pegs covered with hydroxyapatite (HA). When assembled, the pegs are immersed in media. The CBD has several advantages for the development of ex vivo biofilms, which include 96 replicates of a given biofilm, selection of adherent cells, collection of individual pegs and return to incubation, media replenishment and use of different media/conditions (LiPuma et al 2009, Soares et al 2015). The CBDs were incubated in anaerobic and capnophilic atmospheres for up to 16

weeks with 6 different types of media. The best medias used were 1) Trypticase Soy (T-soy) broth supplemented with siderophore, pyoverdines Fe complex and horse blood 2) Columbia broth supplemented with hemin, Vitamin K₃ and horse blood 3) Oxoid heart infusion supplemented with hemin, Vitamin K₃ and horse blood 4) SHI media supplemented with hemin, Vitamin K₃, n-acetylmuramic acid (NAM), urea and sheep blood (Tian et al., 2010) 5) Brain heart infusion (BHI) supplemented with hemin and sheep blood 6) Artificial saliva supplemented with hemin and Vitamin K₃ (Kinniment et al., 1996).

Inocula and biofilms formed on CBD pegs were collected at days 4,10,14, 25, 35 and 55. Microbial analysis was done on the plaque samples, the inoculum, plate growth, CBD peg biofilms, spent media. Duplicate CBD peg biofilms and spent media were collected and frozen at -80°C for future study. Microbial composition was determined using 16S rRNA sequencing (V3-V4 region, MiSeq, Illumina) and relative abundance (% of total reads) were determined using QIIME and HOMINGS. The microbial data generated in this study as well as the microbial samples stored were used to guide, optimize and test the imaging approach proposed here.

1. Identification of uncultured taxa of interest

More than 240 phlotypes have commonly colonize the oral cavity (homd.org). Because it is not feasible to seek them all simultaneously and it is unlikely that they are equally relevant, the most prevalent and numerous phlotypes were chosen. That was accomplished by analyzing the 16S rRNA sequencing data from the 1,311 biofilms and media samples from the previous study.

2. Develop protocol using cultured species

Pure culture cells of known periodontal pathogens *P. gingivalis*, *F. nucleatum* and *A. Israeli* were chosen. These bacteria were selected because of their distinguishable shape which allow for easy identification during imaging and because they are present in periodontal disease. *F. nucleatum* and *A. Israeli* pure culture cells were grown T-soy agar plates supplemented with 5% sheep blood (TSAII) for up to 3 days. *P. gingivalis* were grown T-soy agar plates supplemented with hemin, Vitamin K and 5% sheep blood for up to 3 days.

a) Select probes

The probes that were selected were POGI Cyanine 5 (Cy5) with an excitation spectrum 590-650 nm for *P. gingivalis* [15,16] FUNU Cyanine 3 (Cy3) with an excitation spectrum of 532-558 nm for *F. nucleatum* and EUB338 (FAM) with an excitation of 450-490 nm for all bacteria [16,17]. These probes were chosen because their differences on the excitation spectrum allowed for clear identification when imaging and their use in previous FISH studies. All three probes were purchased from Integrated DNA Technologies, Inc.

b) Test probes for specificity and cross-reactivity:

Once probes were selected and synthesized, they were tested for specificity and cross reactivity, as follows:

-Using pure cultures of reference strains and mixed cultures

Cells were prepared for FISH by starting with the fixation step. Cells were immersed into 1-3ml of phosphate buffered saline (PBS) (optical density (OD) 600nm: 1 and adjusted to 1×10^9 taking into account the size of the bacteria), centrifuged with 10,000(g/rcf) for 5 minutes to form a pellet. The pellet was then immersed in 500 μ l of 4% paraformaldehyde (PFA in PBS (pH 7.4)) and stored in 4°C for at least 2 hours but up to

12 hours. Sample was then centrifuged with 10,000 (g/rcf) for 5 minutes and washed with 500 μ l pre-chilled PBS 3 times, centrifuging in between washes. Sample was then placed into 500 μ l of 50% (v/v) ethanol/PBS solution to permeabilize the cells. Cells were then stored at -20°C.

The hybridization step follows by preparing hybridization buffer containing 25% formamide (the concentration of formamide (10 to 50%) depends on the relationship between probe concentration and fluorescence intensity) with 0.9 M NaCl, 0.01% SDS, 20 mM Tris-HCl (pH 7.5). The probe was dissolved into hybridization buffer, on ice (final concentration: 1 μ M (stock 100 μ M) the concentration is optimized according to the cell number. The sample was then placed into 25 μ l of hybridization solution containing the probes and incubated at 46°C for 4 h (min 2h, up to 6 h). Sample was then immersed in pre-warmed FISH washing buffer (0.2 M NaCl, 0.01% SDS, 20 mM Tris-HCl, pH 7.2, and 5 mM EDTA) and incubated at 46°C for 15 minutes. Cells were then washed 3 times with pre-chilled PBS. Sample can then be stored in PBS at 4°C for up to 3 days before imaging [18]

-Ex vivo biofilms

After confirming the specificity and cross reactivity with the pure culture cells and probes, the biofilm pegs were prepared for imaging using the same protocol with a few modifications. Instead of using a microcentrifuge tube to do the washes and probing the pegs were prepared in a 96 well plate. After the fixation step in formaldehyde, pegs were adhered to the lid of the 96 well plate with orthodontic wax in a column. Starting from the left the pegs were immersed in PBS in the first column, then second column and third column for 30 seconds. The hybridization step was carried out by placing the probe and formamide mixture in the wells so the biofilm on the peg was immersed and incubating the

pegs for 2 hours at 46°C. The pegs were then washed with pre-warmed washing buffer at 46°C 6 times to ensure all unbound probe was removed. Finally, the pegs were washed with pre-chilled PBS 3 times. Pegs were then stored in PBS at 4°C.

c) Optimize lysis, fixation and microscopy protocols

An additional permeabilization step was carried out when working with gram positive bacteria due to their more rigid wall, which includes washing the cells with 500 µl of PBS and then immersing them in lysis solution (10 mg/mL lysozyme in 100 mM Tris-HCl, 5mM EDTA pH = 8). Sample was then incubated for 10 minutes in 37°C and then washed with PBS 3 times. For the same reason fixation time for gram positive pure culture cells was extended and they were fixed overnight to allow permeabilization of the probe.

Imaging of the pure culture cells on glass slides was done with a widefield epifluorescence microscope (Leica DM6000B). Five microliters of the sample were taken and placed on a glass slide with cover slip and viewed under 20x and 100x magnification. Images were then processed using the Leica deconvolution software to get rid of out of focus information and sharpen the image

Imaging of the pegs was done with a confocal microscope (Leica LSM800 and Zeiss LSM 880) using 20x magnification with optical zoom ranging from 1-5x and using z-stack slices from 0.9-2 microns. Like the widefield epifluorescence microscope, the confocal microscope also uses fluorescence, but the difference is that a confocal microscope uses optical sectioning to get better resolution of the fluorescent image and allows for more contrast. Optical sectioning means that it captures multiple two-dimensional images at different depths what we refer as the z-axis, to reconstruct 3D images. Another differences between the two microscopes is the light source, for the epifluorescence microscope it's a mercury lamp and for the confocal it's laser. Another

feature is the pinhole in the confocal which blocks out of focus light in image formation. Even with thicker samples confocal lasers will give better resolution in the z axis, smaller out of focus area and also allow us to get 3D reconstruction. The optical resolution in the z-axis for the confocal microscope is 0.5 μm and 2 - 3 μm for the widefield epifluorescence microscope. We started our imaging experiments with the epifluorescence microscope because we were using pure culture cells on glass slides and were not dealing with a lot of depth but moved to the confocal microscope when imaging the biofilm samples which are much thicker. The peg was stabilized both upright and horizontally with orthodontic wax, placed in a plastic cup and immersed in PBS for imaging.

3) Synthesize and test probes for target; refine protocol

To identify the bacteria of interest, *Megasphaera HOT 123*, probe MEGA123 (Colombo et al. 2009) was added and it replaced POGI (Cy5). Development of probe MEGA 123 was possible by the data provided from Colombo's study, which provided the 16S rRNA sequence for *Megasphaera HOT 123* that could then be used to design a FISH probe which was purchased from Integrated DNA Technologies, Inc. Since *Megasphaera HOT 123* is not a culturable bacteria, in order to check for specificity and cross reactivity two databases were used. Using the Silva database [19] which provides sequences for all three domains of life (Bacteria, Archaea and Eukarya), the sequence gave three genera that had the same sequence somewhere in their genome. To ensure that these results were accurate, the Human Oral Microbiome Database (HOMD) was then checked which focuses on oral bacteria and gives results at the species level. Entering the same sequence gave only one result, *Megasphaera HOT 123*, which assured the use of the probe in the FISH and imaging.

In order to maximize binding and fluorescence intensity of the MEGA 123 probe, the hybridization protocol had to be modified. Using the MathFish program [20] the hybridization efficiency and formamide curves were generated. The formamide concentration was adjusted from 25% to 10%, which provides a range where both MEGA 123 (Alexa 647) and EUB338 (Alexa 488) can bind to target sequence. The protocol was further modified with the substitution of EUB338 (FAM) with EUB338 (Alexa 488) which is a more stable probe. The probe concentration for all probes was diluted from 1 μ M to 0.1 μ M. Also, to ensure the hybridization buffer and probes were fully removed from the samples, the number of washes was increased from 3 to 6.

4) Employ protocol on stored pegs (replicates)

Biofilm pegs with *Megasphaera HOT 123* were prepared for FISH using a 96 well plate in the same manner that the test pegs had been prepared. Pegs were probed with MEGA 123 (Alexa 647) to identify target bacteria, EUB338 (Alexa 488) to identify all bacteria and FUNU (Cy 3) to identify *F. nucleatum*. The probe concentrations used were 0.1 μ M and the number of washes were 6 after the hybridization step.

CHAPTER 4: RESULTS

1. Determine the uncultured taxa of interest

We chose the taxa of interest by looking through all the sequencing data, 1311 samples from 16 patients, and selected the taxa in both peg biofilms and media that were in the highest proportions (Table 1). We then narrowed it down to the taxa that were in the top 10% which were *Megasphaera Human Oral Taxon (HOT) 123*, *Prevotella HOT 526*, *Prevotella HOT 315*, *Aggregatibacter HOT 898* and *Alloprevotella 912*, the one that was chosen for imaging was *Megasphaera HOT 123*.

The reason for selecting *Megasphaera HOT 123* as our target is that it had the highest number of samples in the top 10% when we ranked the most prevalent taxa in the biofilm and media samples. Having at our disposal all the sequencing data and prevalence at the five different time points allowed us to choose exactly what biofilm or media sample to image to look for our target.

2. Develop protocol using cultured species

1. Select probes

The first step was to assess the specificity and cross reactivity of our probes, FUNU for *F. nucleatum*, POGI for *P. gingivalis* and EUB338 for all Eubacteria using pure culture cells. When imaging FUNU with *F. nucleatum*, POGI with *P. gingivalis* and EUB338 with *F. nucleatum* we noted their specificity as they were all fluorescently labeled when viewed with the epifluorescence microscope (Fig. 1a-c). Also noted at this time was that the EUB338 and POGI probe bleached out when imaged, the EUB338 probe more than the POGI.

2. Test probes for specificity and cross-reactivity using:

- Mixed cultures

Next pure cultures of mixed cells, *F. nucleatum*, *P. gingivalis* and *A. israelii* were prepared for FISH and imaged with widefield epifluorescence to test for cross reactivity and lysing protocol. Including *A. israelii*, a gram positive bacteria, meant we had to perform the additional lysing step in the protocol, therefore we had to test if lysing all bacteria together led to a different result compared to lysing *A. israelii* first and then combining it with the other gram negative bacteria and continuing the hybridization protocol. The images showed no difference, which reassured us that all bacteria could be lysed together in similar experiments on slides but also on the pegs, which have a combination of gram positive and gram negative bacteria (Fig. 2a-b). Also noted was that there was no cross-reactivity between the probes, FUNU labeled *F. nucleatum*, POGI labeled *P. gingivalis* and EUB338 labeled all eubacteria.

- Ex vivo biofilms

Having tested the probes on pure culture cells, the biofilm pegs were prepared next for FISH and imaging using both the widefield epifluorescence and confocal microscopes. We started with the epifluorescence microscope and noted that under the highest magnification we could use, 20x, we were not able to see individual cells, but we can see that the probes were working (Fig. 7). Using the same peg we imaged with the confocal microscope with 20x magnification and similarly we were not able to see individual cells (Fig. 8a) but the advantage of this microscope over the epifluorescence is the ability of optical magnification, which allowed us to get better resolution and 3D reconstruction (Figure 8b). Using then optical zoom ranging from 1-5x we were able to

start seeing some individual bacteria (Fig. 9). But the amount of *P. gingivalis* noted was higher than expected; it did not seem to correlate with the amount present in the peg, which was 3.6%. A possible explanation was the HA on the peg or bacteria had autofluorescence. Also noted was the inability to see all the individual cells stained with the EUB338 probe, it appeared to be a high amount of extracellular matrix (ECM). Using a curette some of the biofilm was removed and placed on a slide to see if more individual bacteria could be identified, but no difference was seen compared to imaging the peg (Fig. 10). In order to see if there was autofluorescence, we prepared a peg following the usual FISH protocol except for the addition of the probes. The images revealed some autofluorescence in all 3-probe excitation spectrums (Fig. 11). Therefore, the next step was to check if the intensity of our probes was high enough to distinguish between the autofluorescence and the fluorescence from our probes. Using the same peg, it was once again prepared with the same protocol and similar images were observed, what appeared to be *P. gingivalis* was higher than expected (Fig. 12). The next step was then to check the autofluorescence of HA, a peg without biofilm was imaged with the confocal microscope. The image showed autofluorescence in the POGI (Cy5) spectrum, which explained the high level of what was thought to be *P. gingivalis* (Fig. 13)

- Optimize lysis, fixation and microscopy protocols

When imaging the mixed cultures, *P. gingivalis* and *F. nucleatum* what was not clearly seen in the same quantity was *A. israelii*. Comparing the differential interference contrast (DIC) images to the fluorescent images allowed us to see that the bacteria were present but not as high in numbers as the other bacteria and not always labeled with the EUB338 probe (Fig. 3). Analyzing the images led us to two possible explanations for this. One was that we did not have the same amount of *A. israelii* cells compared to *P. gingivalis*

and *F. nucleatum* because we had not corrected for cell size when calculating OD. *A. israelii* cells are much bigger bacteria and therefore a higher number of them are needed to have equal volumes compared to the other two bacteria. The second explanation was that the probe had not been able to penetrate the cells, since gram positive bacteria are known to have a much more rigid cell wall compared to gram negative bacteria. To resolve this issue the cells were fixed overnight (19 hours).

The same experiment was ran with the two corrections, one sample fixed for 4 hours and one fixed for 19 hours both of them with corrected cell size. The images for the sample fixed for 19 hours and with cell size correction allowed us to better see *A. israelii* (Fig. 4). What was also noted was that *A. israelii* had some autofluorescence as it was seen under the POGI (Cy5) excitation spectrum when it should have only been seen under the EUB338 (FAM) spectrum (Fig. 5a-b). To rule out autofluorescence with the other bacteria they were all ran through the FISH protocol except for the addition of the probes. The images showed slight autofluorescence from *A. israelii* and *F. nucleatum* (Fig. 6a-d).

3) Synthesize and test probes for target; refine protocol

To resolve the problem with autofluorescence both from the bacteria and HA, the FISH protocol was modified. There was substitution of EUB338 (FAM) with EUB338 (Alexa 488) which is a more stable probe. To look for the target bacteria, *Megasphaera HOT 123*, the probe MEGA123 (Alexa 647) was added and it replaced POGI (Cy5). The concentration of the probe was decreased from 1 μ M to 0.1 μ M. To make sure that the unbound probe was not getting trapped in the ECM, the number of washes in the protocol was also increased. The images were then clearer, there was less background autofluorescence and the EUB338 (Alexa 488) probe did not bleach as quickly (Fig. 14a-c).

4) Employ protocol on stored pegs (replicates)

Both media from pegs that had *Megasphaera HOT 123* and the pegs themselves were prepared for FISH. In total throughout this project, 18 biofilm pegs and 10 media were probed (Tables 2 & 3). The media was prepared for FISH with EUB338 (Alexa 488) and MEGA 123 (Alexa 647) probes and imaged using the epifluorescence microscope. The image showed specificity and no cross reactivity between the two probes. All bacteria were labeled with EUB338 (Alexa 488) and only *Megasphaera HOT 123* was labeled with MEGA 123 (Alexa 647). *Megasphaera HOT 123* appears to be a coccoid and the cells appear to cluster (Fig. 15a-b). Similar results were seen when a peg was imaged (Fig. 16).

To prove that our images were in fact true and accurate, we prepared another sample of media collected from pegs, which contained *F. nucleatum*. The media was prepared for FISH with EUB338 (Alexa 488) and FUNU (Cy3) probes and imaged with epifluorescence microscope. The image showed specificity and no cross reactivity between the two probes, FUNU stained *F. nucleatum* and EUB338 (Alexa 488) stained all bacteria (Fig. 17).

To further validate our results and see if our images of *Megasphaera HOT 123* could be replicated another peg was prepared and imaged using the confocal microscope (Fig. 18). The peg was probed with the EUB338 (Alexa 488) and MEGA 123 (Alexa 647) and what was observed what appeared to be the *Megasphaera HOT 123* cocci, but the biofilm thickness prevented the clarity previously seen in the other images. To evade this problem, it was decided that imaging media would be the best option and more samples of media containing the target bacteria were imaged. The images allowed us to distinguish individual bacteria and observe more clearly the cocci shape of *Megasphaera HOT 123* (Fig. 19 & 20). Our results showed that the media was giving us consistent images of our

target bacteria, but one problem we encountered was that we were losing a lot of our sample in our preparation. This then led us to try ultra-stick slides (ThermoFisher Scientific) which are glass slides with an adhesive coating which allows us to do our FISH protocol right on the slide. A test was first ran using a pure culture of *F. nucleatum* probed with FUNU probe and imaged with the epifluorescence microscope. The results were successful because we were able to conserve more of our sample as well as get similar quality images (Figure 21&22). At this point we were ready to try this method with our target bacteria and samples were probed using EUB338 (Alexa 488) for all bacteria in green and MEGA 123 (Alexa 647) for *Megasphaera HOT 123* in red. The images showed individually labeled bacteria that allowed us to see the cocci shape of *Megasphaera HOT 123* (Fig. 23a &b).

To further test our protocol and our probes on the slides we added our FUNU probe to detect *F. nucleatum* for which we used a blue filter for, we kept our EUB338 (Alexa 488) probe in green which detects all bacteria and our MEGA 123 (Alexa 647) in red Fig. 24a&b). Finally, in order to verify that there was no cross-reactivity with our MEGA 123 (Alexa 647) probe we prepared slides that had no *Megasphaera HOT 123* using the ultra-stick slides. We found media that contained plenty of other bacteria, but no *Megasphaera HOT 123* by looking through our sequencing data (Table 4). Imaging was done using the epifluorescence microscope and probing with EUB338 (Alexa 488) for all bacteria in green and MEGA 123 (Alexa 647) for *Megasphaera HOT 123* in red. The images showed the EUB338 (Alexa 488) probe staining all eubacteria, but when looking at the red filter there was no fluorescence indicating that our MEGA 123 (Alexa 647) was specific for *Megasphaera HOT 123* (Fig. 25&26).

CHAPTER 5: DISCUSSION

The rationale for identifying the spatial relationships and distribution of uncultured periodontal microorganisms in periodontitis is that it will provide information about their location within the biofilm and relationships with other bacterial species. Collectively, this knowledge will shed light onto their metabolism, their growth requirements and potential pathogenic role in periodontal diseases. In order to accomplish that, it is critical to develop a protocol that will allow the study of the biogeography of candidate periodontal pathogens so that ultimately, it is possible to study these organisms, their pathogenic mechanisms and whether there can be an useful to guide diagnosis, treatment and monitoring of periodontal patients.

Sequencing approaches uncovered the magnitude of their presence in the oral cavity [8,9,21,22]. By cataloguing the most commonly found uncultured oral taxa, the Human Microbiome Database (HOMD) identified 240 phylotypes that frequently detected in the oral cavity. However, it is not feasible of productive to pursue the cultivation of all of them. They are unlikely to be equally prevalent or relevant regarding periodontal diseases.

That rationale is supported by the study of Perez-Chaparro et al [10]. The authors performed a systematic review of the literature to determine the weight of evidence of association studies on candidate periodontal pathogens. Out of the hundreds of phylotypes found in the published studies, the authors identified a subset that seemed to be more consistently associated with periodontal diseases. That type of strategy can be useful in selecting which phylotypes merit further pursuit.

We performed a similar strategy in the present study. In order to develop a method to image uncultured organism, it is important to determine that they are frequent enough

to be detected and studied. In order to do that, we screened 18 ex vivo biofilm samples and 10 biofilm-derived media samples to determine the prevalence and relative abundance of the most common uncultured taxa. We sought the 240 phylotypes listed in HCMD. We observed that *Megasphaera* HOT 123, *Prevotella* HOT 526, *Prevotella* HOT 315, *Aggregatibacter* HOT 898 and *Alloprevotella* HOT 912 were the most prevalent and commonly detected at levels of at least 10% of the sample. In order to facilitate the development of the method, we selected *Megasphaera* HOT 123, which had a prevalence between 5-67% in 113 of the samples.

One of the challenges in developing methods to study uncultured organisms is that their cells are not readily available. Hence, often times, the method has to first be devised using cultivated, reference strains of organisms found in the same environment. That strategy has been used in the past. In the study of Teles et al [23], the authors developed the RNA-oligonucleotide quantification technique (ROQT). The ultimate goal of ROQT was to enumerate uncultured organisms in oral biofilm samples. In testing the best approaches to extract RNA from cells, select probes, determining their specificity and sensitivity and hybridization conditions, pure and mixed cultures of reference strains of oral bacterial species were employed.

In the present study we hypothesized that, investigating the ecological relationships of uncultured taxa with the environment and other members of the community in situ could give important insights into the metabolism of uncultured organisms and ultimately, facilitate their isolation and cultivation. A similar rationale is found in the study by Mark Welch et al [14]. The authors used Combinatorial Labeling and Spectral Imaging FISH (CLASI-FISH) to study the biogeography of supragingival plaque. The authors described a cauliflower structure, where the perimeter was composed of

Streptococcus and *Haemophilus/Aggregatibacter* on the distal tips of *Corynebacterium* filaments, what she calls the foundation or anchor of the biofilm. Just below that, in the annulus, there are *Fusobacterium* and *Leptotrichia*. This arrangement tells us that these bacteria are benefitting from each other, what can be identified as helper species. The bacteria that are facultative anaerobes like *Streptococcus* use the tips of *Corynebacterium* to be near oxygen, sugar and saliva as a nutrient source. While the *Fusobacterium* and *Leptotrichia* are near the streptococci, which create the anaerobic environment, they thrive in [14].

A step forward then is to be able to describe what Mark-Welch described for supragingival plaque for uncultured bacteria in subgingival plaque. Therefore, it is of critical importance to develop a protocol to identify the spatial relationships and distribution of uncultured periodontal microorganisms in periodontitis ex-vivo biofilms through imaging.

In our study, we capitalized on the existence of curated 16S rRNA sequences to be used as probes and on the fact that bacterial rRNA is ubiquitous and abundant in biofilms. And our study has shown that the development of a probe for an unculturable bacteria using 16s rRNA sequence, preparing pegs or their media for FISH, followed by imaging with confocal and epifluorescence microscopes can guide us in the direction to identify these bacteria in subgingival biofilms.

Having both an epifluorescence and confocal microscope at our disposal we were able to fully take advantage of what each has to offer. Although both microscopes use fluorescence and allow us to view our fluorescently labeled bacteria, we learned that the epifluorescence worked best with our pure culture and media samples. This is because these samples were imaged on glass slides and there is not much depth to them. While the confocal microscope was better when imaging our peg biofilms because of the

thickness of the biofilm as well as the actual size of the peg. Unlike the epifluorescence microscope, the confocal microscope has optical sectioning which allows for 3D reconstruction of much thicker samples, which was perfect for our biofilm pegs. Both microscopes have filter sets for FAM, Cy3 and Cy5 which allowed for the simultaneous use of our three probes. Probes that were selected because they would be easily discernable from one another due to their excitation spectrum differences. Even when making the substitution of our EUB338-FAM probe for EUB338 (Alexa 488) and when adding the MEGA123 (Alexa 647) probe for our target bacteria there was a clear distinction in their light emission.

In the process of identifying our target bacteria, *Megasphaera HOT 123*, we noted that we had better quality images when imaging media, but one issue was that we lost a large portion of our sample in the preparation process. In order to address the issue, we switched our FISH preparation of media samples from microcentrifuge tubes to ultra stick slides. These slides have an adhesive coating which allows the preparation to be done on the slides. Probing with EUB338 (Alexa 488) and MEGA123 (Alexa 647) allowed us to identify *Megasphaera HOT 123* in the media samples and see that it's cocci in shape.

Megasphaera HOT 123 belongs to the genus *Megasphaera*, family Veillonellaceae, class Clostridia, phylum Firmicutes. The genus comprises Gram-stain-negative, obligately anaerobic bacteria [24]. So far, only five species of the genus *Megasphaera* have been described [25,26]. The type species *Megasphaera elsdenii* [24] is found in the rumen of cattle and sheep and in the feces and intestine of humans and pigs [24,27,28,29,30,31,32]. *Megasphaera cerevisiae*, *Megasphaera paucivorans*, and *Megasphaera sueciensis* are brewery-associated species [33,34], while *Megasphaera micronuciformis* was recovered from a human liver abscess and a pus sample [35].

Species phylotypes belonging to this genus have been identified, catalogued and sequenced as part of the Human microbiome project. In samples from the urogenital tract, *Megasphaera elsdenii*, *Megasphaera genomosp. type_1* str. 28L, *Megasphaera sp.* BV3C16-1 and *Megasphaera sp.* UPII 199-6 were identified. Two strains of *Megasphaera micronuciformis* were identified, one in urogenital samples and one in the oral cavity.

The recent sequencing study by Nallabelli et al [36] detected *Megasphaera* in the oral cavity of a healthy individual. In the subgingival plaque sample collected from the subject, *Megasphaera sp.* strain DISK18 was identified. The authors described it as an anaerobic, gram negative non-motile cocci which can form pilus like appendages during the initial phase of biofilm development. What was noted was the absence of virulence factors like collagenase which is observed in other periodontal pathogens like *P. gingivalis*. They hypothesized that it is an early colonizer because of its coaggregation with *Streptococcus* and *Lactobacillus* which are pioneer oral colonizers.

Other studies have looked at Indian populations, both healthy and those with gastrointestinal diseases. A study comparing the gut microbiome between those with celiac disease to their first-degree relatives without the disease and controls found that the duodenal microbiota of celiac disease subjects had higher abundance of amplicon sequence variants from genera *Megasphaera* and *Helicobacter* [37]. Kulkarni then looked at healthy subjects to determine the core microbiome and its metabolic role. When looking at the most prevalent genus they found *Prevotella*, *Bacteroides*, *Faecalibacterium*, *Dialister* and *Megasphaera*. They also found that the major biochemical pathways contributed to carbohydrate metabolism, which can be attributed the carbohydrate rich Indian diet [38].

Few studies report on the characteristics of cultivated *Megasphaera* species, especially in human-associated samples. Marx et al [39] described the characteristics of

Megasphaera elsdenii, a gram negative coccus which is found in cattle, sheep and other ruminants. It can produce volatile fatty acids and can relieve acidosis in livestock with the uptake of lactic acid. While Marchandin et al [35] described *Megasphaera micronuciformis* which was isolated from a liver abscess and a pus sample in two subjects. The authors described the bacteria as gram negative anaerobic cocci, usually single cells, 0.4-0.6 μm in diameter, non-motile and not forming endospores.

Lanjekar et al [40] proposed a new species of *Megasphaera*, named *Megasphaera indica*. The strain was isolated from the feces of two healthy subjects residing in India. The bacteria were described as non-motile, anaerobic gram negative cocci with a mean size of 1.4-2.5 μm , occurring singly or in pairs or as short chains. They were described as using carbohydrates like glucose as a nutrient source resulting in formation of volatile fatty acids.

In summary our study has shown the development of a protocol that will allow the study of the biogeography of candidate periodontal pathogens. With the development of a probe using 16s rRNA sequence, preparing pegs and their spent media for FISH, followed by imaging with confocal and epifluorescence microscopes we were able to locate *Megasphaera* HOT 123. We were able to note that it is cocci in shape and can be found in single cells or clusters.

Our findings will advance the field with the use of this protocol by providing information about their location within the biofilm and relationships with other bacterial species. With the ultimate goal being the isolation of these uncultured bacteria so we can study them, their pathogenic mechanisms and virulence factors to develop an efficient guide to diagnosis and treatment of patients with periodontitis.

Future directions include locating the other unculturable bacteria that were in the top 10% of our sequencing data results by using ultra stick slides and imaging with

epifluorescence microscope. As well as identifying the location of the uncultured bacteria within the biofilm by trying other methods of preparation which include embedding the biofilm pegs and sectioning them so that we may easily view individual bacteria while avoiding the thickness of the biofilms.

TABLES

Species Name	Taxa	Number of Samples in Top 10%
Prevotella	315	5
Fretibacterium	361	0
Fretibacterium	362	0
Stomatobaculum	373	0
Seelenomonas	478	0
Prevotella	526	9
Aggregatibacter	898	3
Alloprevotella	912	1
Kingella	12	0
Megasphaera	123	113
Prevotella	305	0
Prevotella	309	0

Table 1. Taxa in the highest proportions. Taxa in red are the ones in the top 10%

Peg #	<i>F. nucleatum</i>	<i>P. gingivalis</i>	<i>Megasphaera</i> HOT 134
1	15.6	0.1	0.0
2	3.6	3.8	0.0
3	2.4	0.0	0.0
4	29.0	2.7	0.0
5	16.7	3.2	0.0
7	4.5	0.0	0.0
8	53.1	1.7	0.1
9	1.6	0.0	0.0
10	24.4	0.5	0.0
11	1.3	0.4	0.0
12	1.4	7.7	0.0
13	2.0	6.4	0.0
14	2.5	70.2	0.0
15	4.3	6.2	0.0
16	8.0	0.0	26.4
17	3.5	0.0	25.9
18	0.0	0.1	26.4

Table 2. Peg Sequencing Data

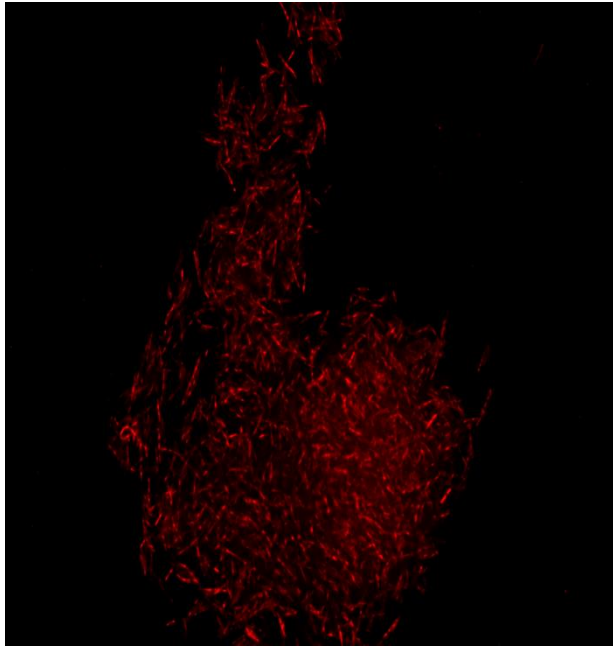
Media	F. nucleautm	P. gingivalis	Megasphaera HOT 123
1	21.83	26.32	12.23
2	24.61	0.00	0.00
3	26.56	9.78	0.00
4	31.23	0.01	0.00
5	32.91	0.00	0.01
6	21.83	26.32	12.22
7	1.45	0.73	6.23
8	26.84	5.41	13.98
9	42.25	0.00	0.00
10	16.92	0.96	0.00

Table 3. Media Sequencing Data

Bacteria	Media 9	Media 10
<i>Megasphaera HOT 123</i>	0.00	0.00
<i>Fusobacterium</i>	42.25	16.92
<i>Veilonella</i>	29.66	1.65
<i>Leptotrichia</i>	14.78	0.00
<i>Selenomonas</i>	4.88	0.00
<i>Gemella</i>	3.87	0.26
<i>Streptococcus</i>	0.15	9.41
<i>Neisseria</i>	0.08	49.68
<i>Aggregatibacter</i>	0.00	7.40
<i>Treponema</i>	0.00	1.98
<i>Eikenella</i>	0.08	0.03

Table 4. Media sequencing data for testing cross-reactivity of MEGA 123 probe

FIGURES



Figures 1a. *F. nucleatum* with FUNU probe (red).

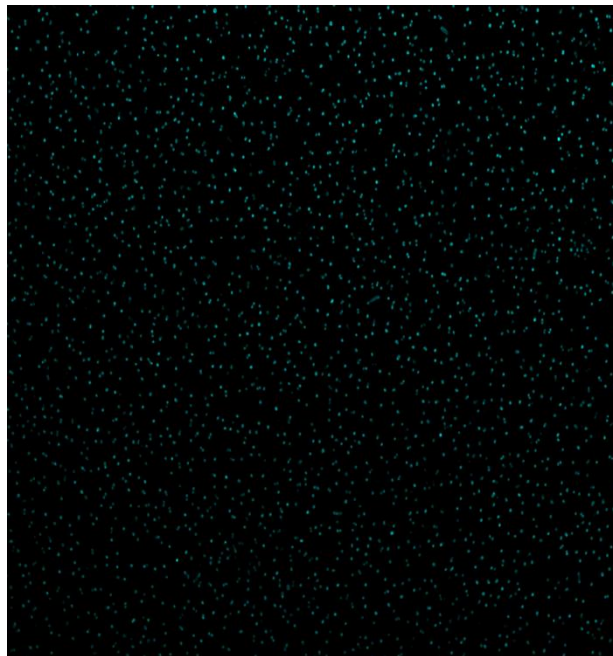


Figure 1b. *P. gingivalis* with POGI probe (blue)

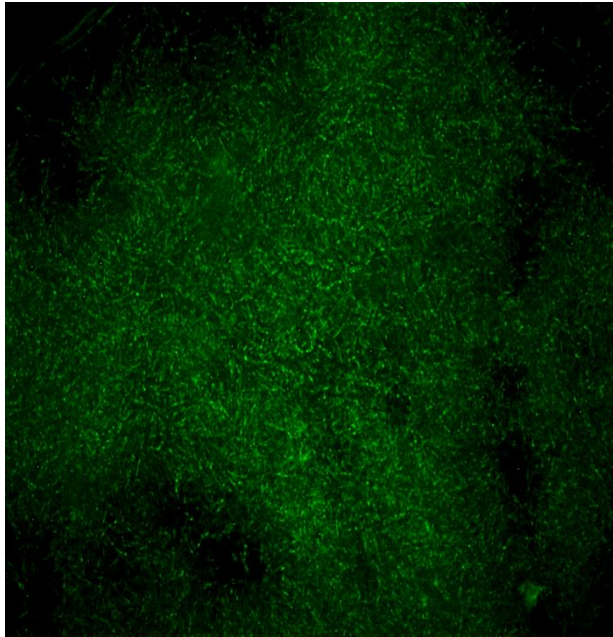


Figure 1c. *F. nucleatum* with EUB probe (green).

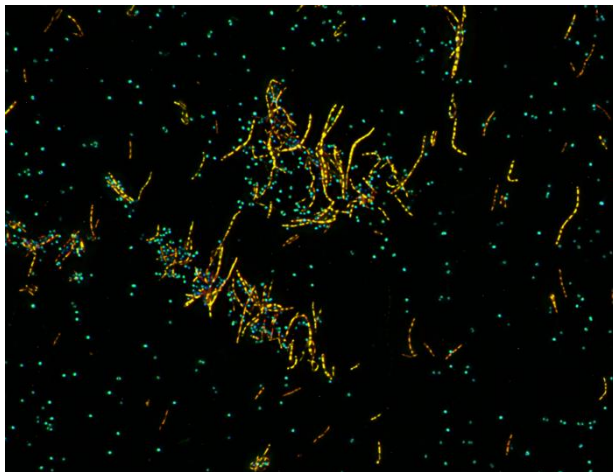


Figure 2a. Lysed together-*F. nucleatum* with FUNU probe (yellow due to combination of EUB in green with FUNU in red), *P. gingivalis* with POGI probe (blue) and *A. israeli* with EUB probe (green).

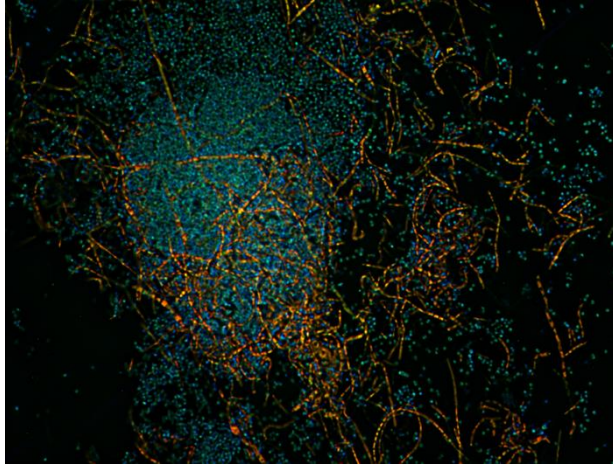


Figure 2b. Lysed separately-*F. nucleatum* with FUNU probe (yellow due to combination of EUB in green with FUNU in red), *P. gingivalis* with POGI probe (blue) and *A. israeli* with EUB probe (green).

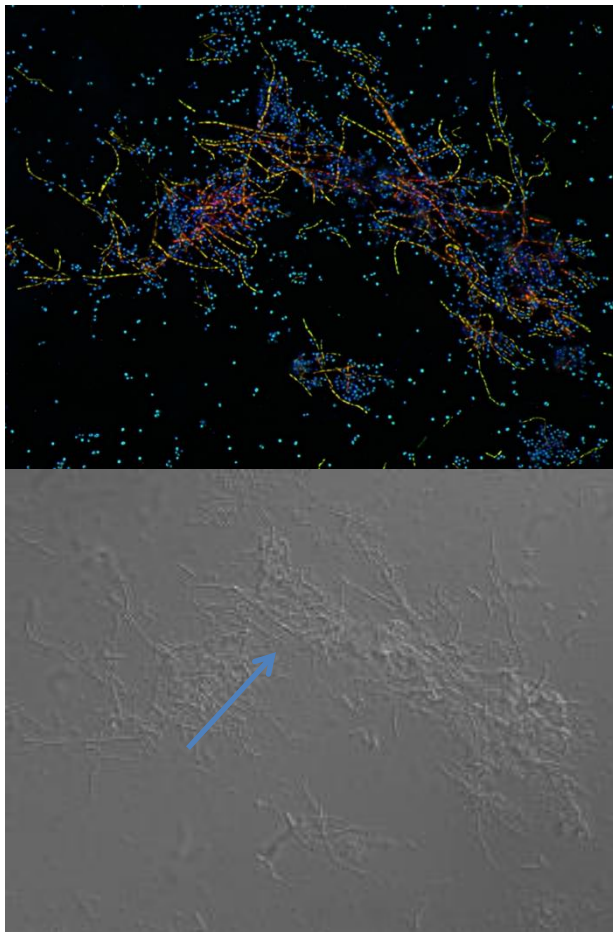


Figure 3. Top image: Lysed together and fixed for 4 hours-*F. nucleatum* with FUNU probe (yellow due to combination of EUB in green with FUNU in red), *P. gingivalis* with POGI

probe (blue) and *A. israeli* with EUB probe (green). Bottom image: DIC showing that not all *A. israeli* (filament shape) were fluorescently labeled.

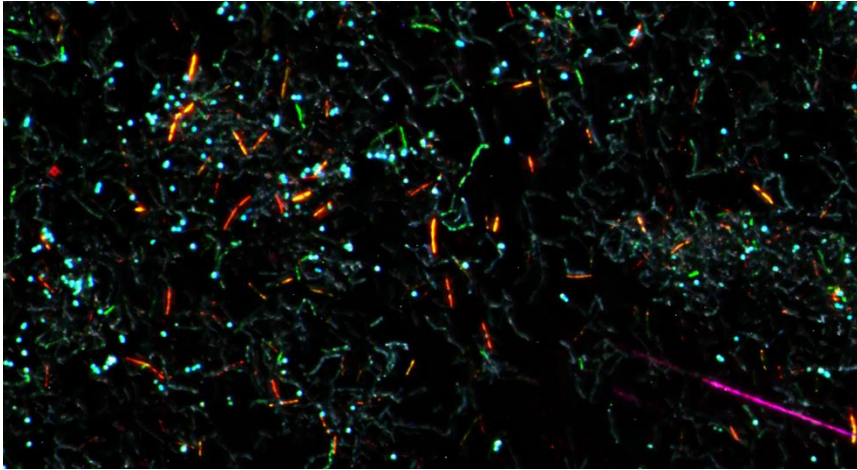


Figure 4. Lysed together and fixed for 19 hours-*F. nucleatum* with FUNU probe (red) *P. gingivalis* with POGI probe (blue) and *A. israeli* with EUB probe (green)

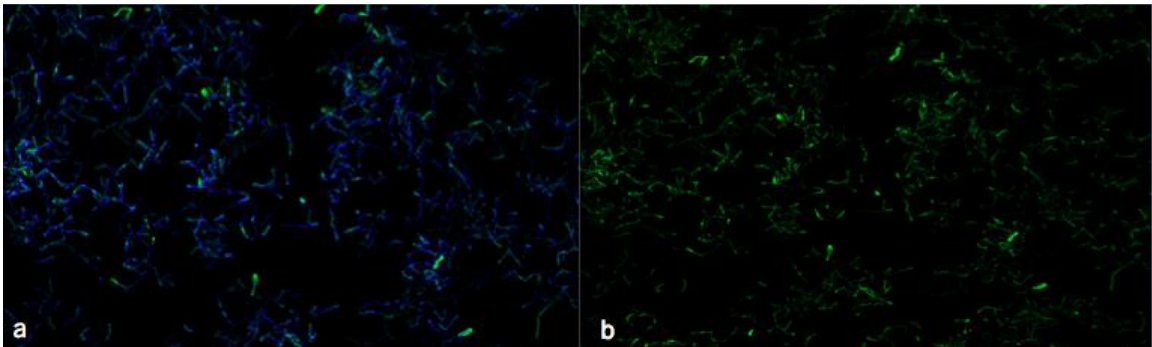


Figure 5a. *A. israeli* showing autofluorescence in Cy5 spectrum. 5b. *A. israeli* with EUB probe (green)

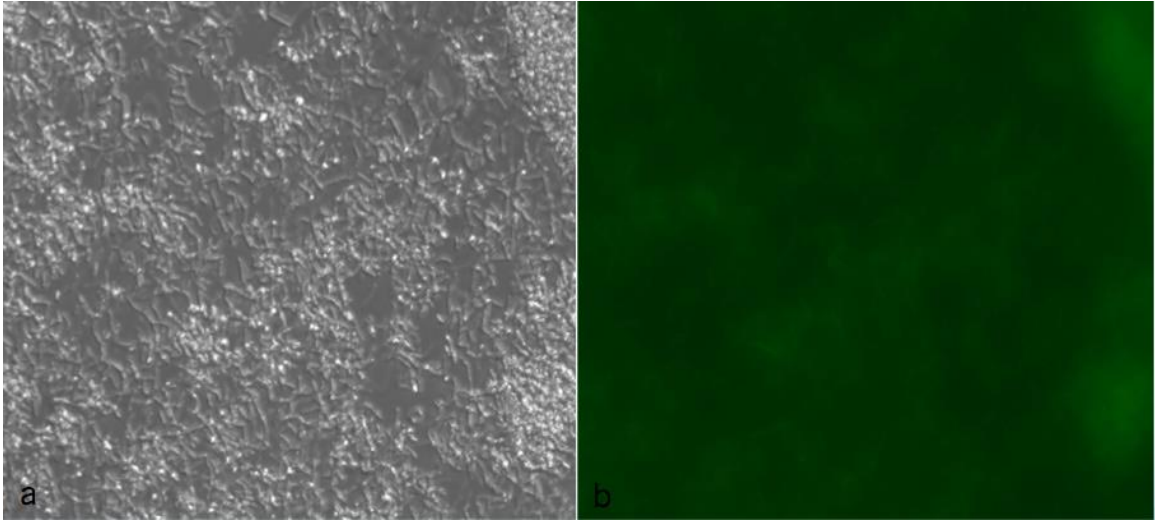


Figure 6a. DIC image of *A. Israeli*. 6b. *A. Israeli* showing slight autofluorescence.

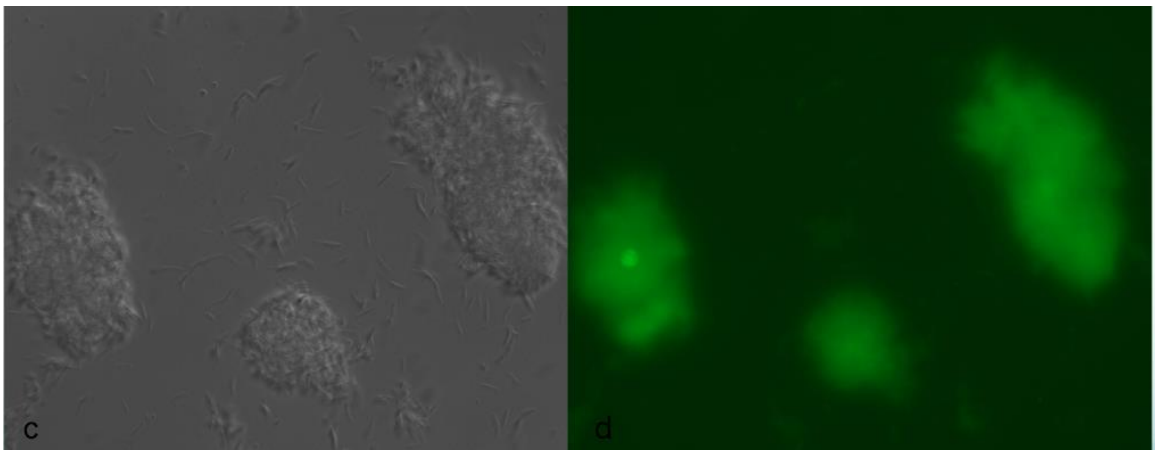


Figure 6c. DIC image of *F. nucleatum*. 6d. *F. nucleatum* showing slight autofluorescence.

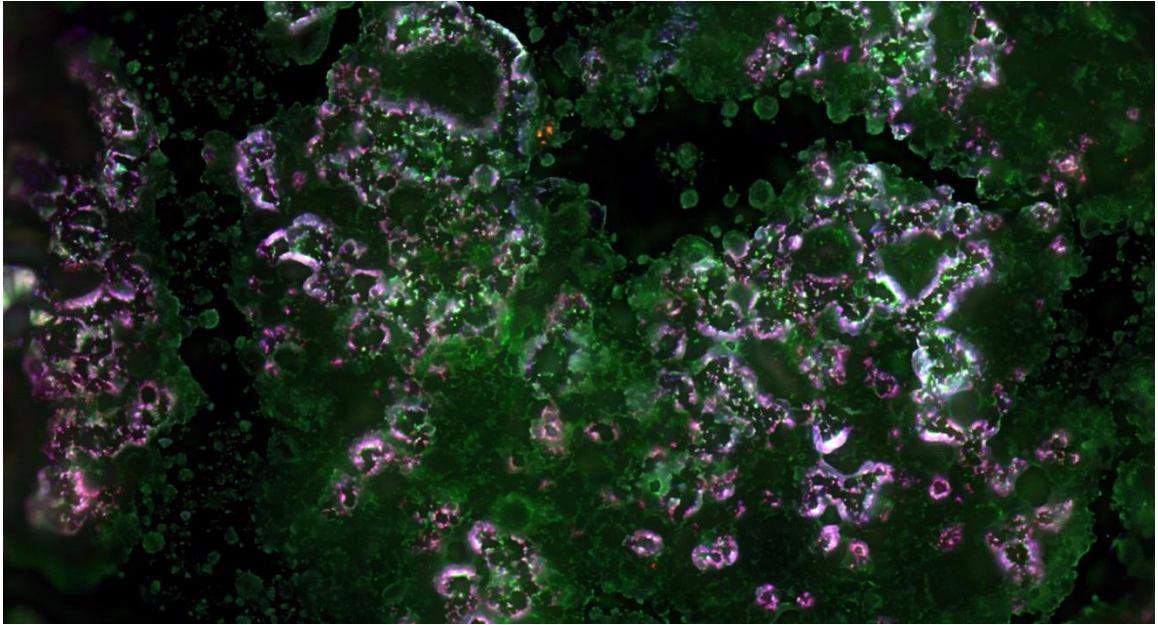


Figure 7. Peg with epifluorescence microscope. *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green). Overlay of FUNU and POGI probes are seen as a magenta color.

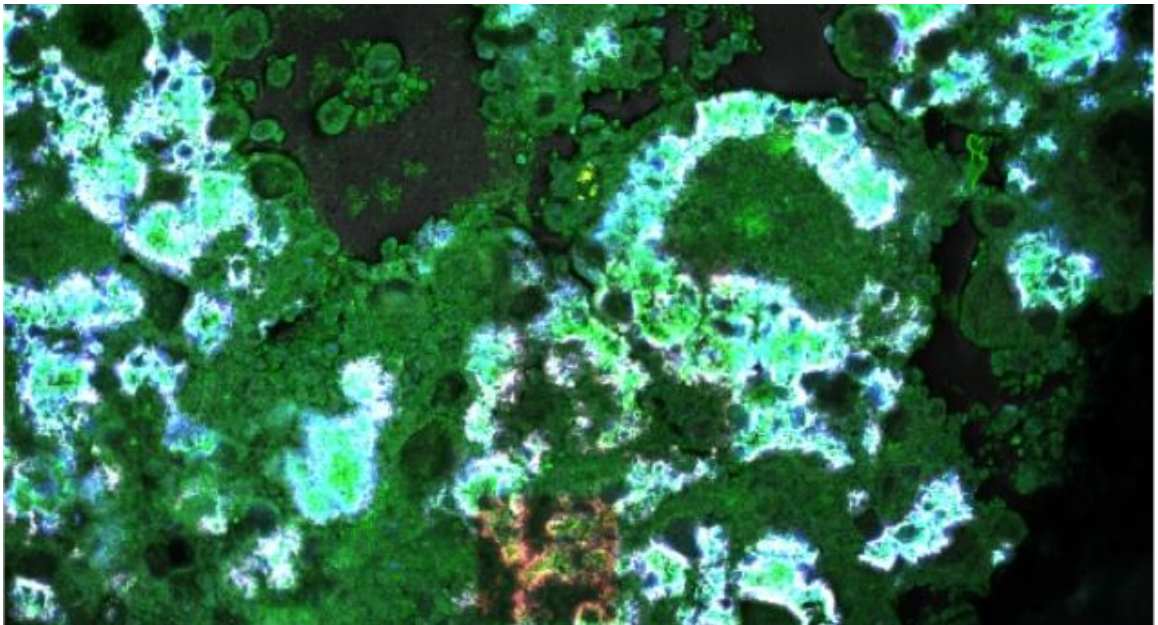


Figure 8a. Peg with confocal microscope at 20x magnification. *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green). Combination of FUNU and EUB probes are seen in yellow.

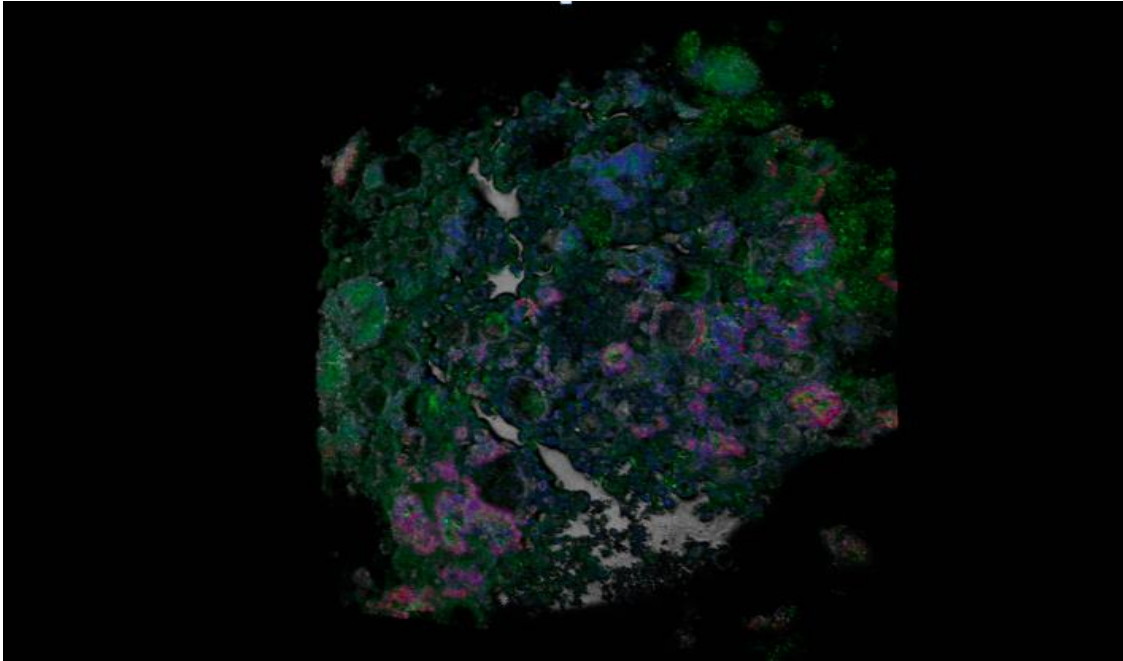


Figure 8b. 3d reconstruction of peg with confocal microscope at 20x magnification. *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green). Overlay of FUNU and POGI probes are in magenta.

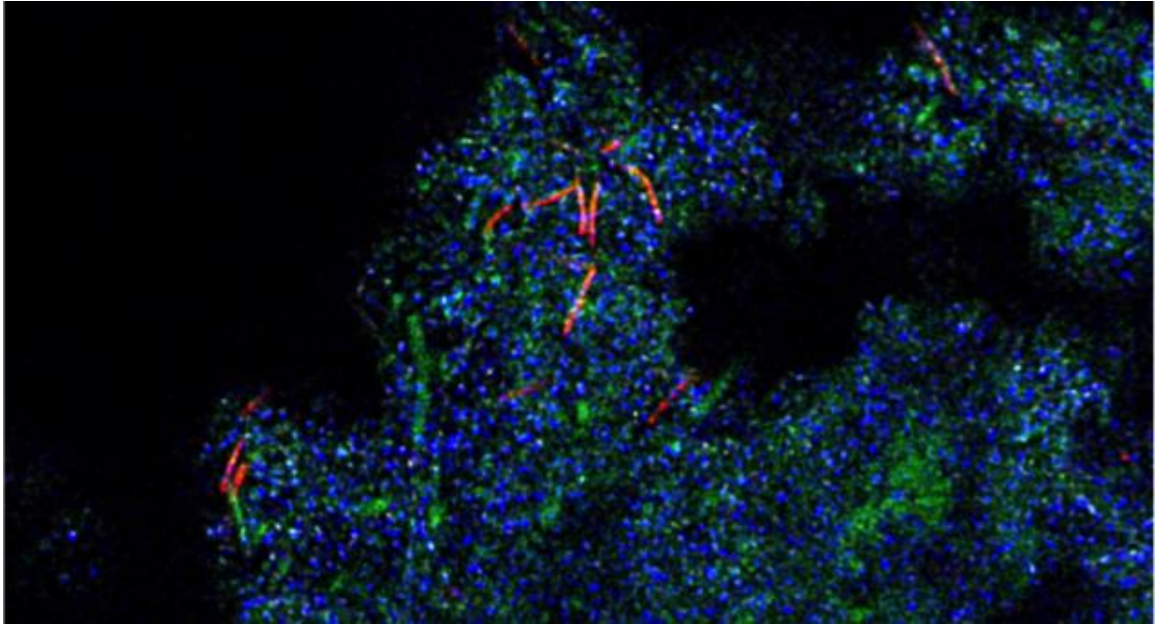


Figure 9. Peg with confocal microscope with optical zoom. *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green).

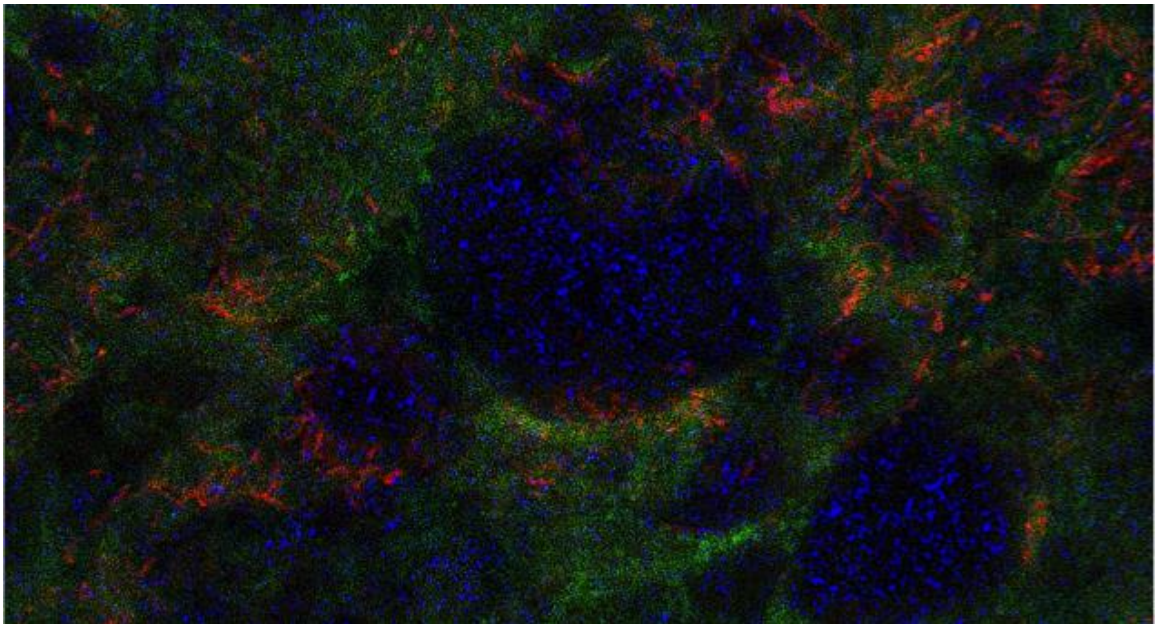


Figure 10. Biofilm removed from peg with curette with confocal microscope and optical zoom. *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green).

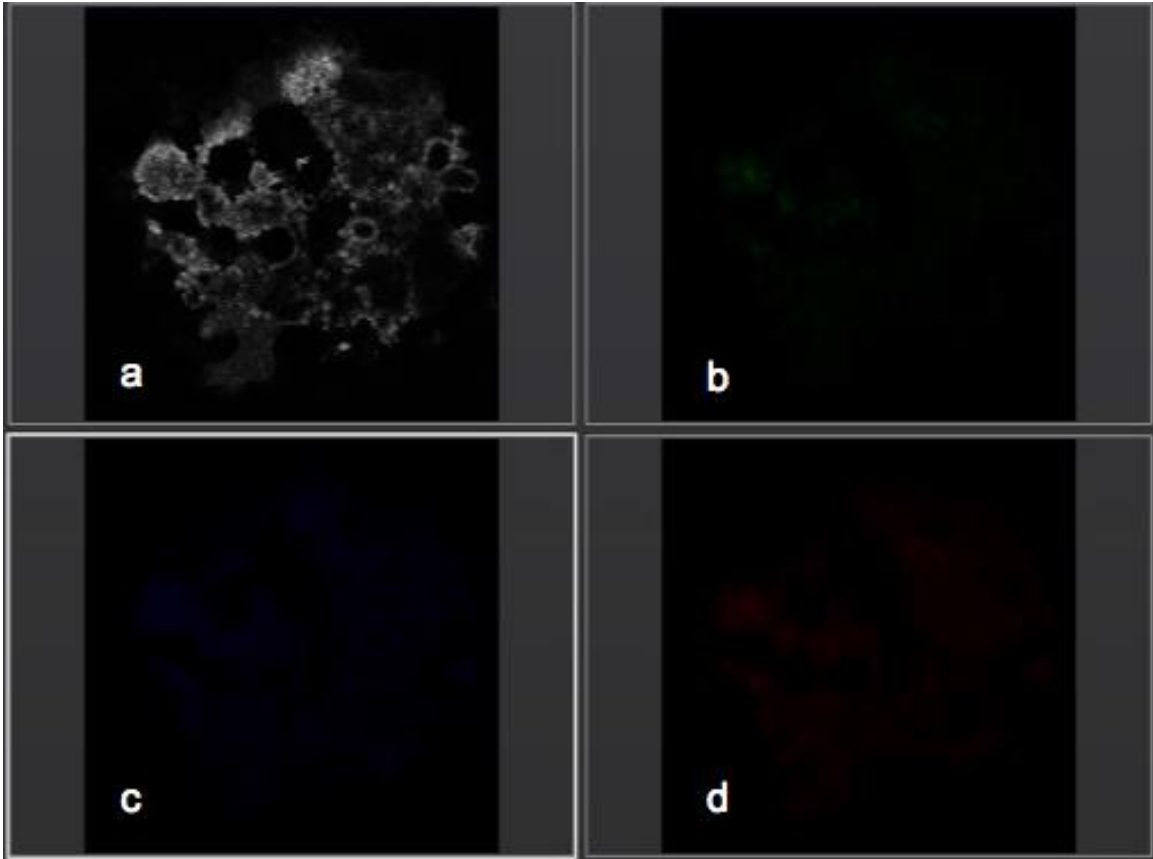


Figure 11. Peg with confocal microscope with no probes. A: DIC image. B: Slight autofluorescence in EUB (FAM). C: POGI (Cy5). D: FUNU (Cy3) spectrums.

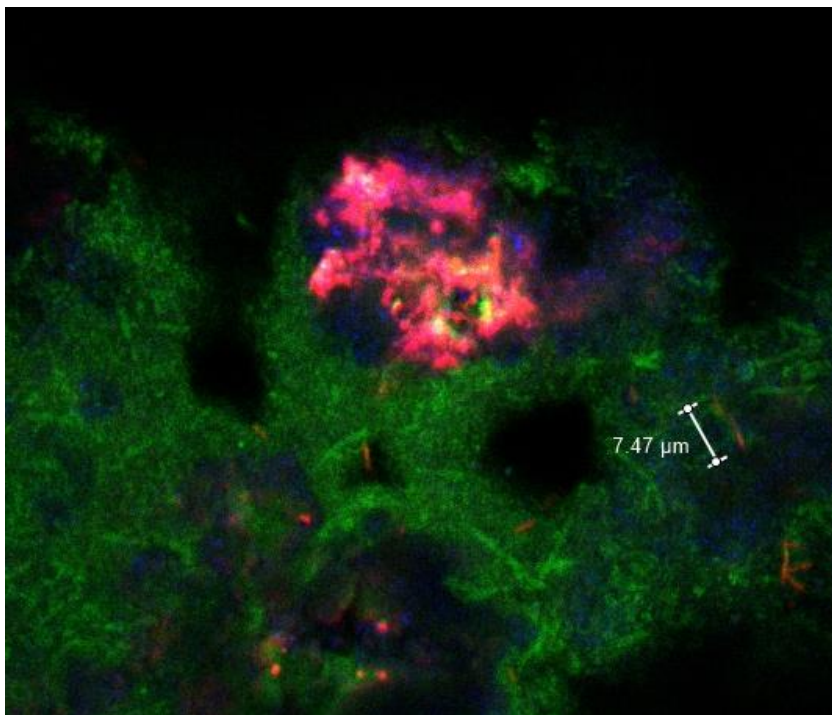


Figure 12. Peg from Figure 11 with the addition of *F. nucleatum* with FUNU probe (red), *P. gingivalis* with POGI probe (blue) and all other eubacteria with EUB probe (green).

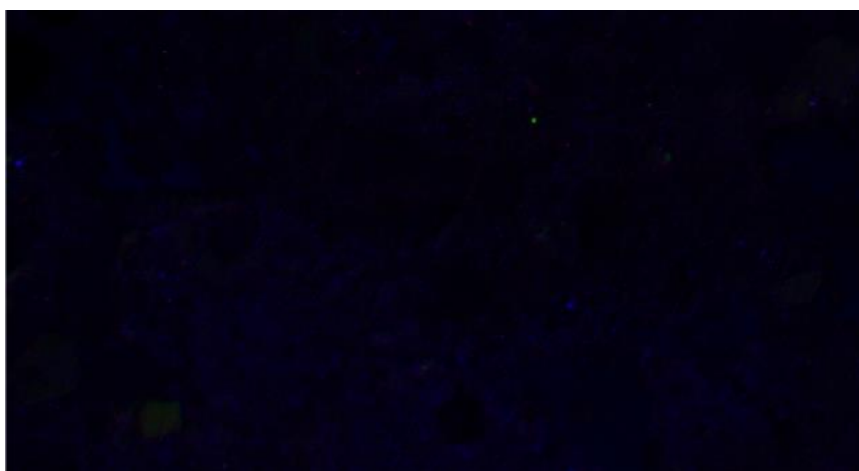
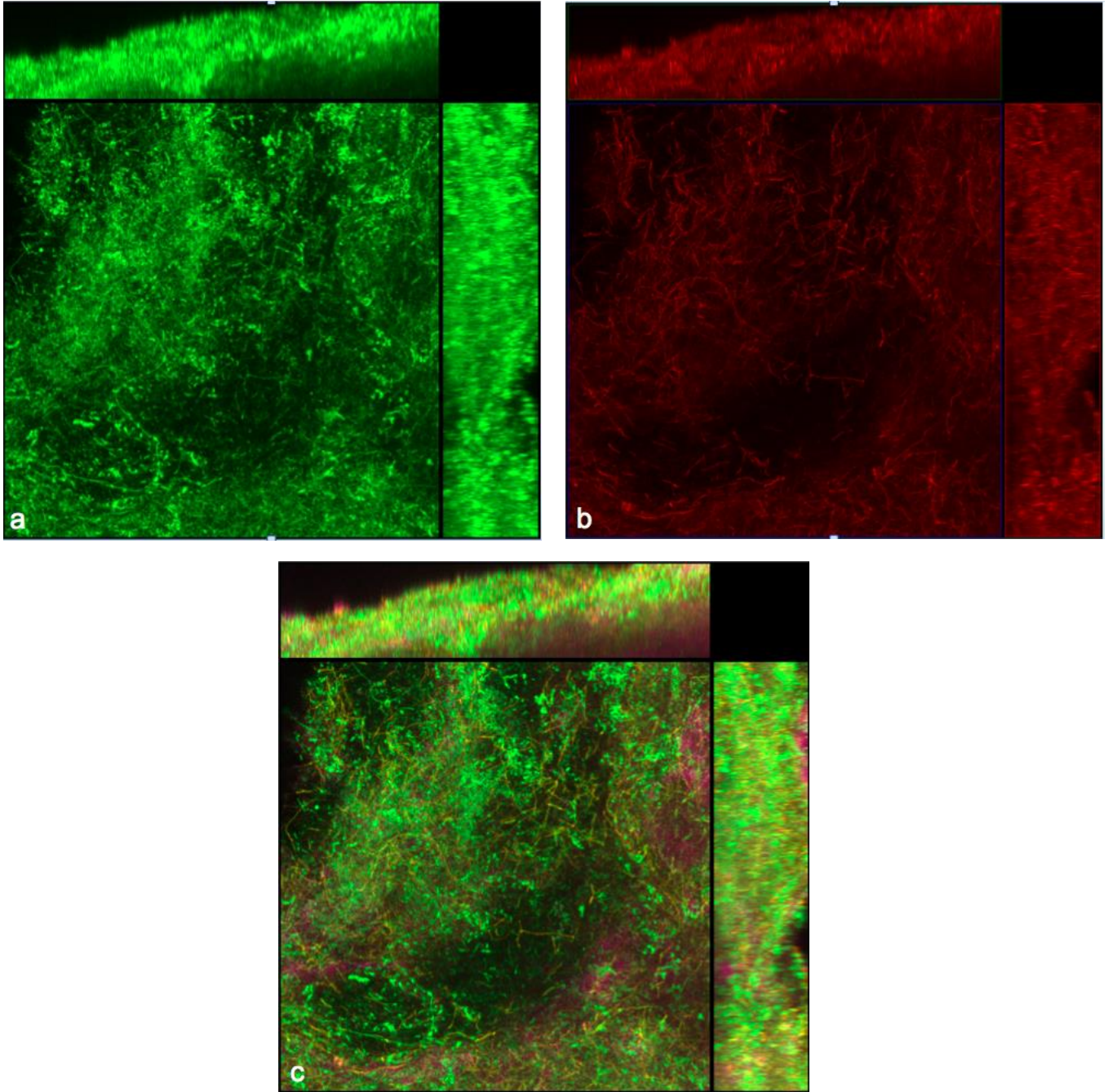


Figure 13. Peg without biofilm showing HA has autofluorescence in POGI (Cy5) spectrum.



Peg 14a. Peg with EUB338 (Alexa 488), 0.1 μM probe concentration and increased washes. A: EUB probe for all eubacteria (green). B: FUNU probe for *F. nucleatum* (red). C: EUB, FUNU and POGI probe for *P. gingivalis* (magenta).

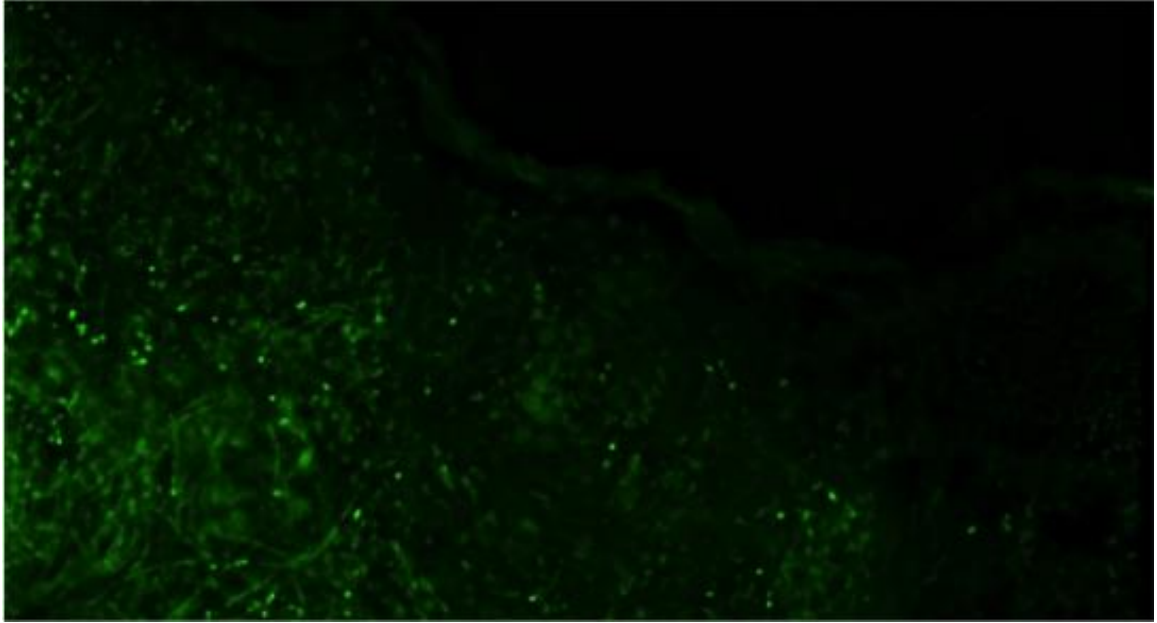


Figure 15a. Media containing *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria.

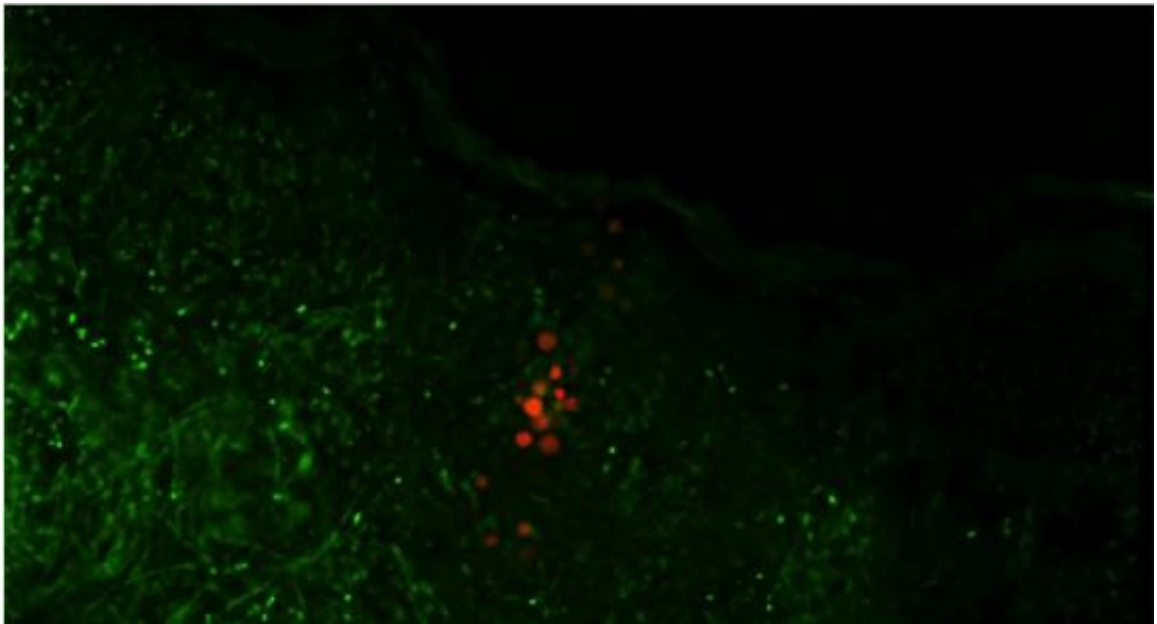


Figure 15b. Media containing *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red.

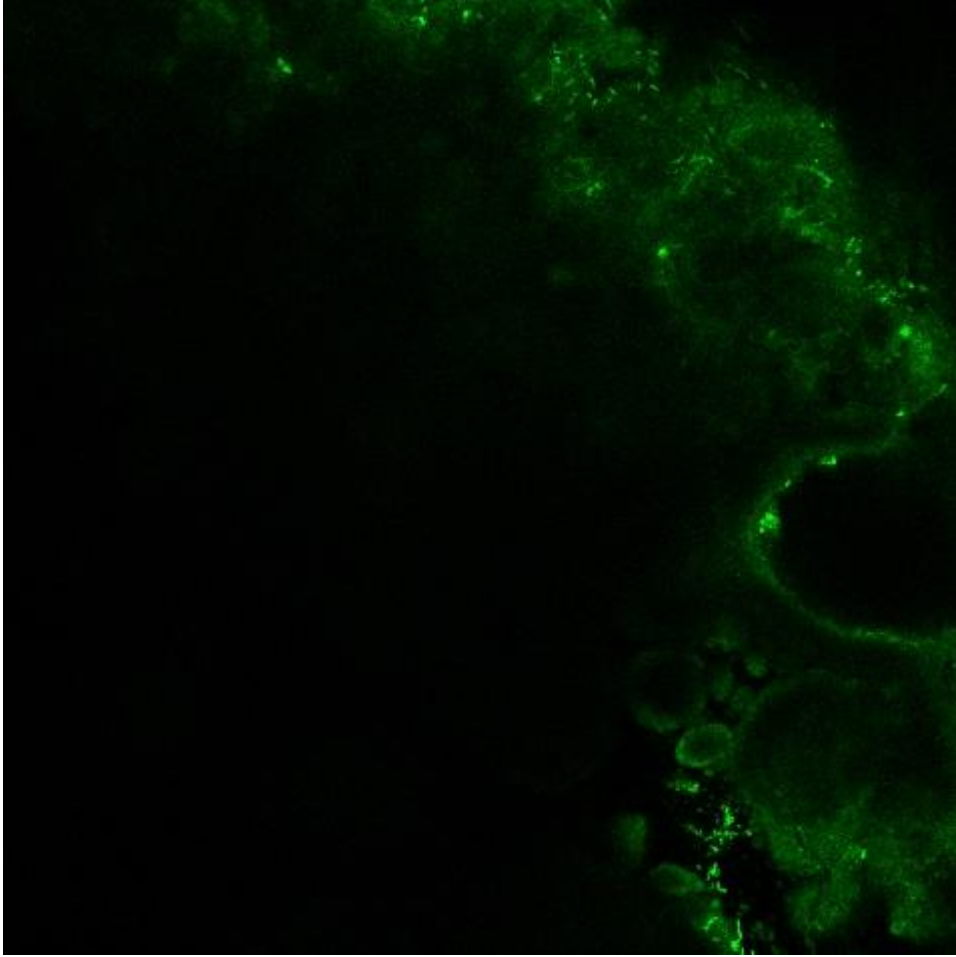


Figure 16a. Peg with *Megasphaera HOT 123*. EUB338 (Alexa 488) probe for all bacteria in green.



Figure 16b. Peg with MEGA 123 (Alexa 647) probe staining *Megasphaera* HOT 123 in red.

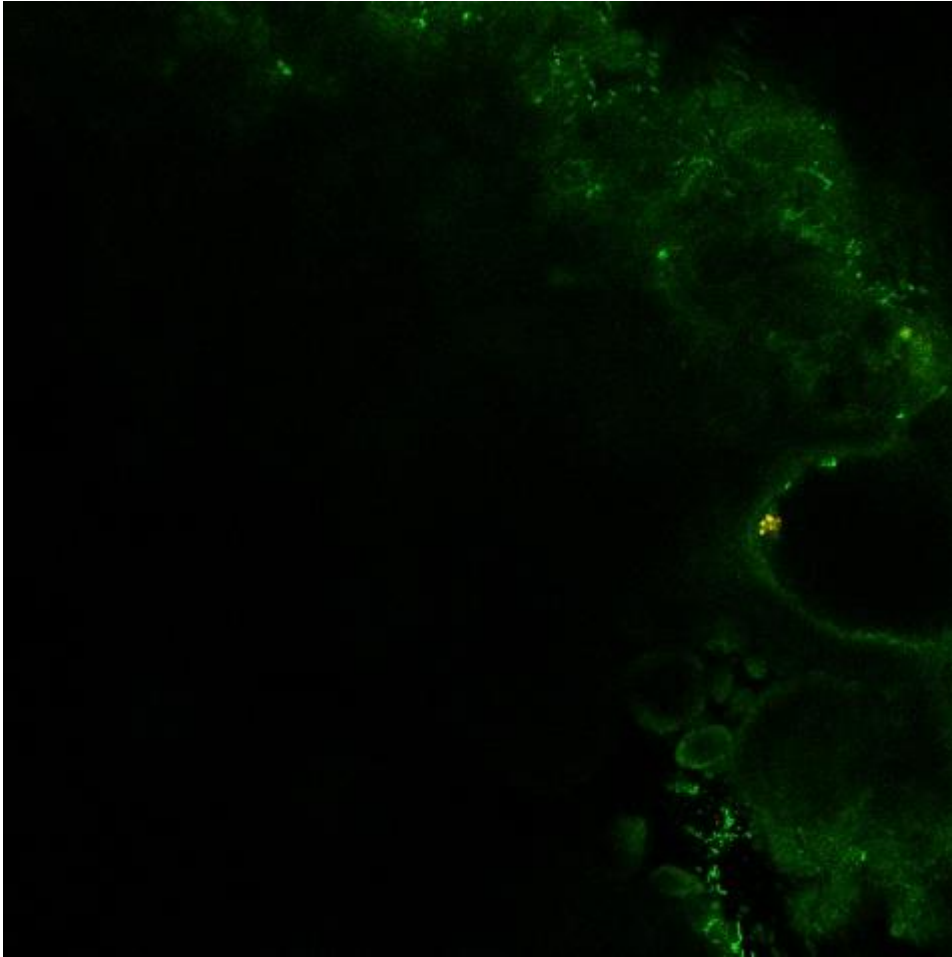


Figure 16c. Peg with *Megasphaera HOT 123* in yellow the result of the combination of EUB338 (Alexa 488) for all bacteria in green and MEGA 123 (Alexa 647) for *Megasphaera HOT 123* in red.

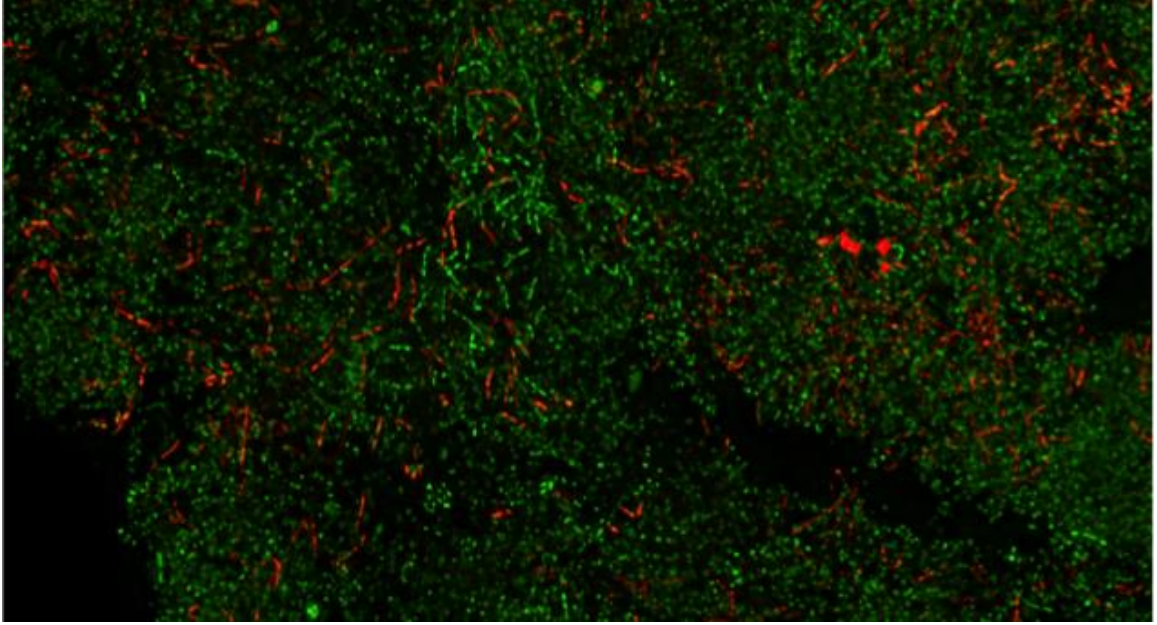


Figure 17. Media containing *F. nucleatum* stained with FUNU probe (red) and EUB338 (Alexa 488) for all bacteria (green).

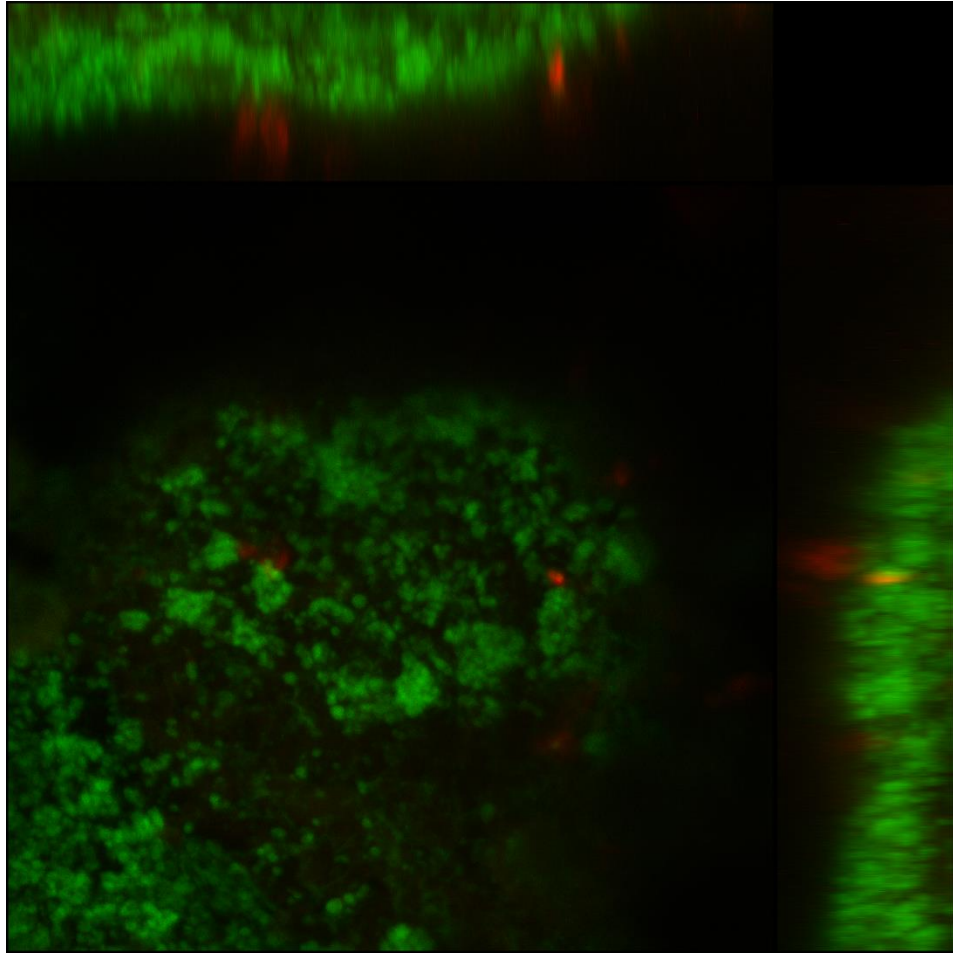


Figure 18. Peg containing *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red.

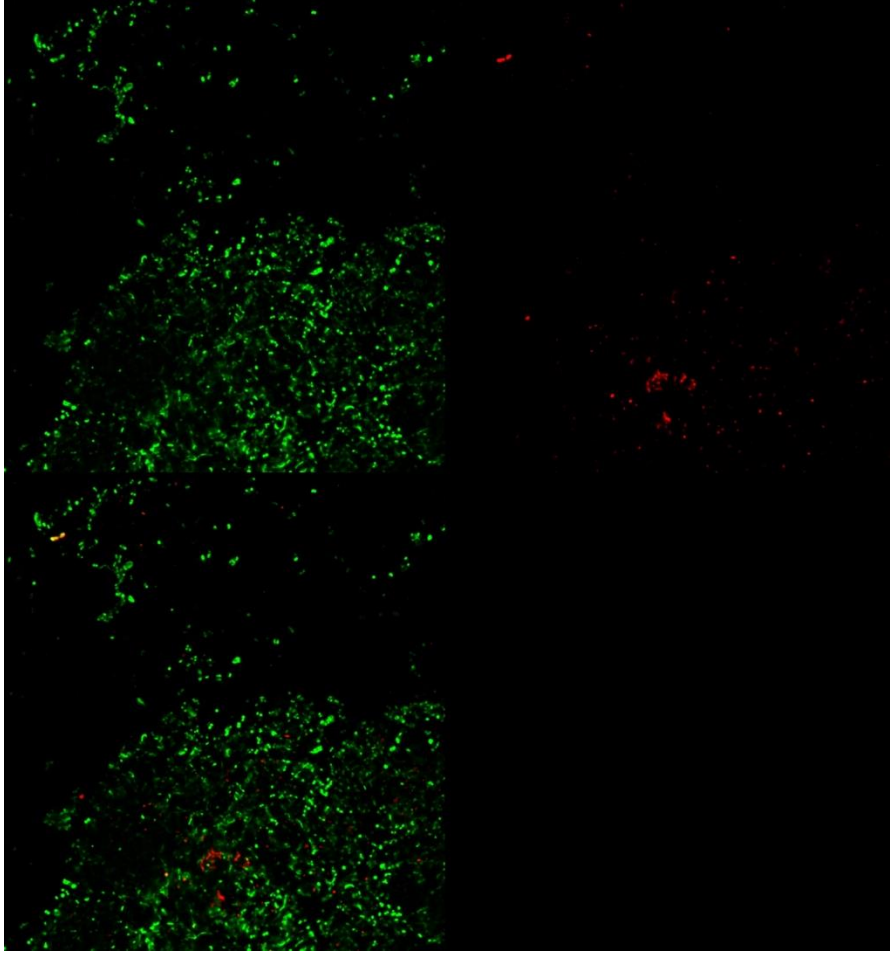


Figure 19. Media containing *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red. Top left EUB338 alone, top right MEGA 123 alone and bottom right shows both EUB338 and MEGA 123 probes.

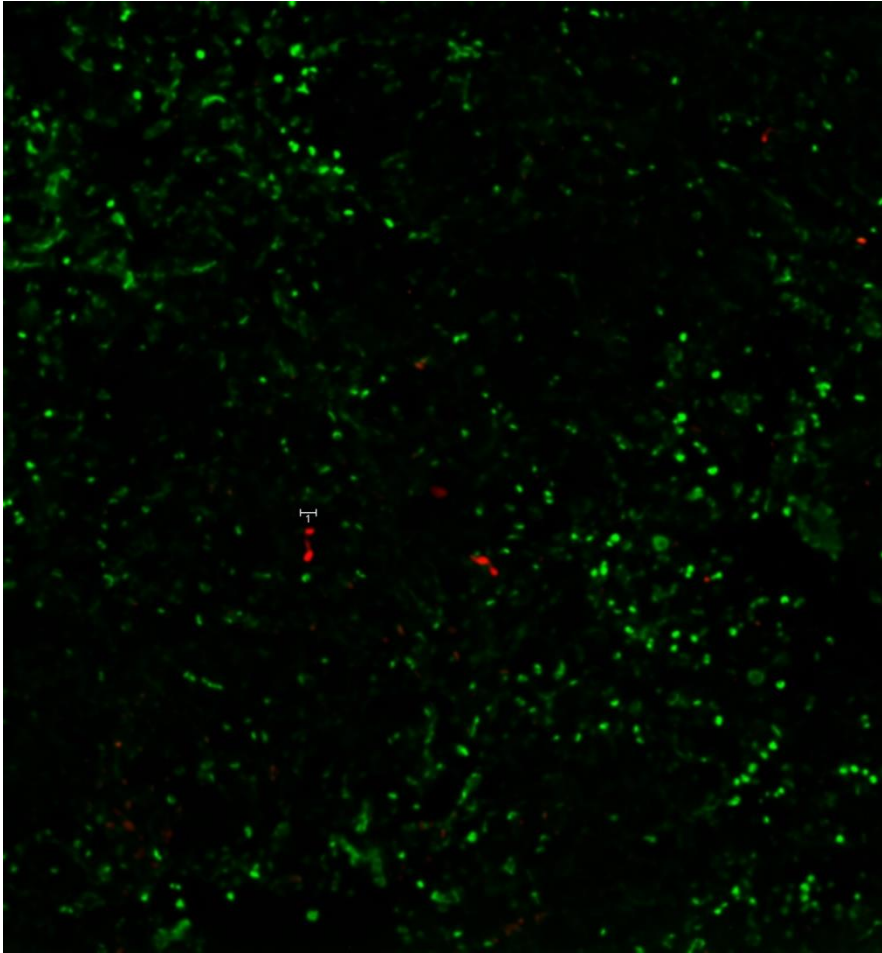


Figure 20. Media containing *Megasphaera HOT 123* with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red. Top left EUB338 alone, top right MEGA 123 alone and bottom right shows both EUB338 and MEGA 123 probes.

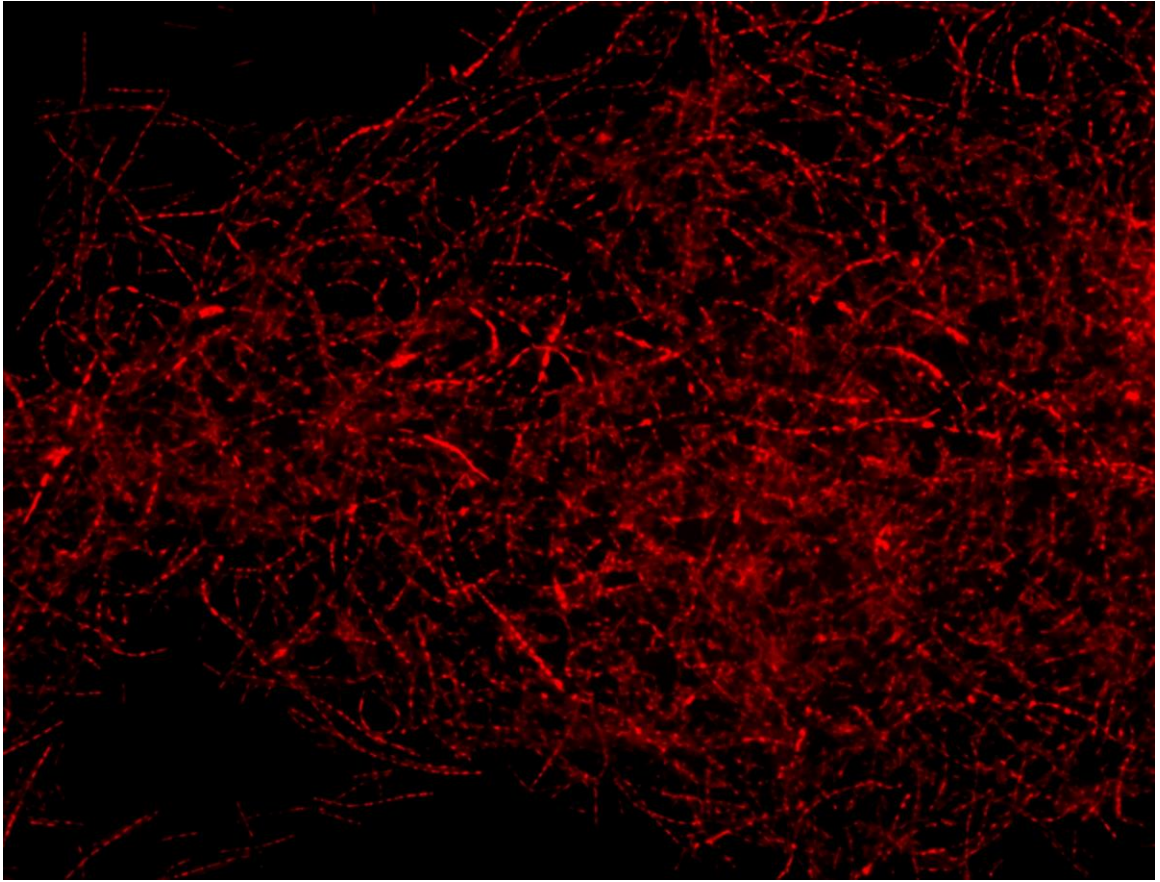


Figure 21. *F. nucleatum* pure culture probed with FUNU probe (red) on Ultra Stick slides.

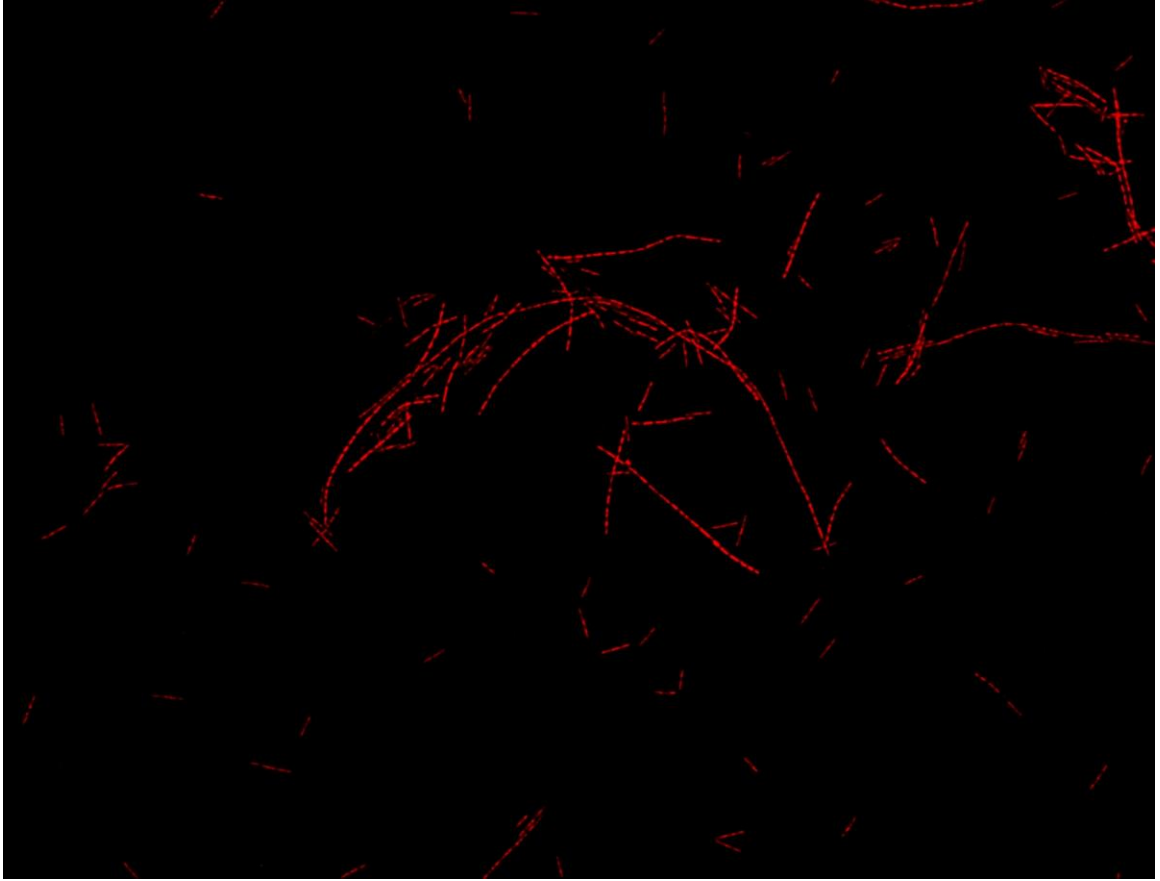


Figure 22. *F. nucleatum* pure culture probed with FUNU probe (red) on Ultra Stick slides.

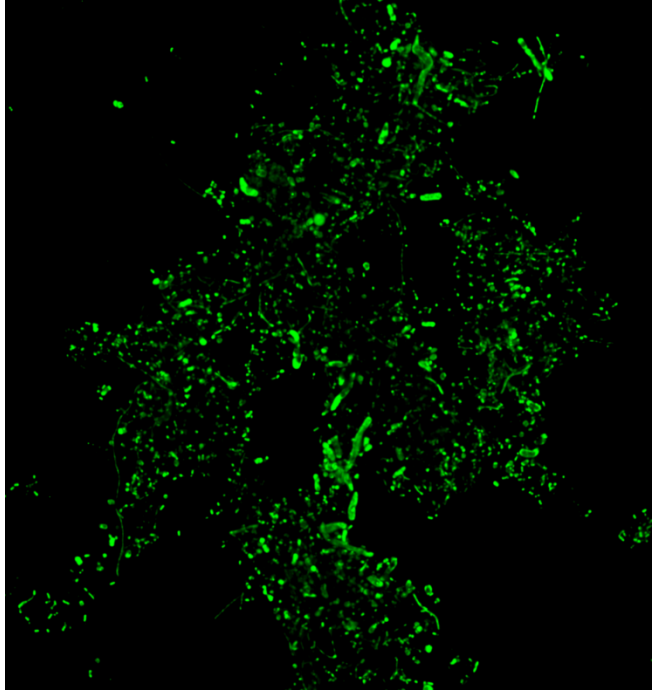


Figure 23a. Media containing *Megasphaera HOT 123* with EUB338 (Alexa 488) probe in green for all eubacteria on Ultra Stick slides.

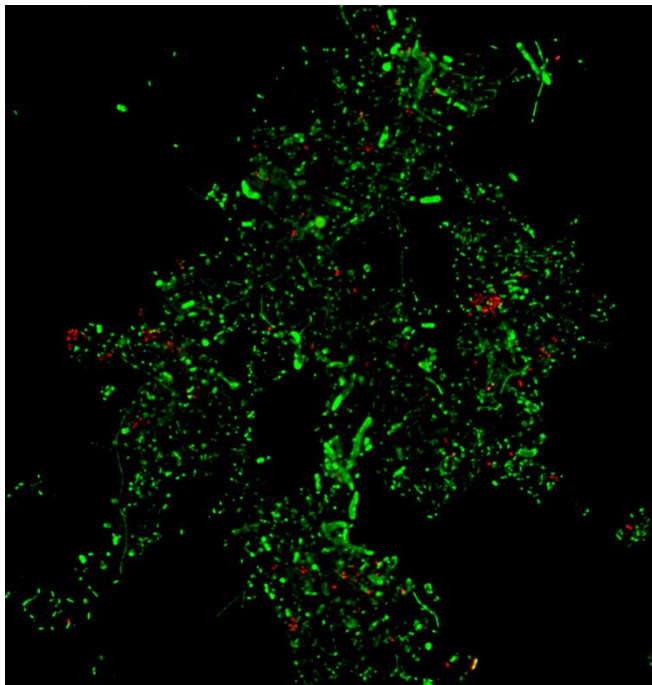


Figure 23b. Media containing *Megasphaera HOT 123* with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red on Ultra Stick slides.

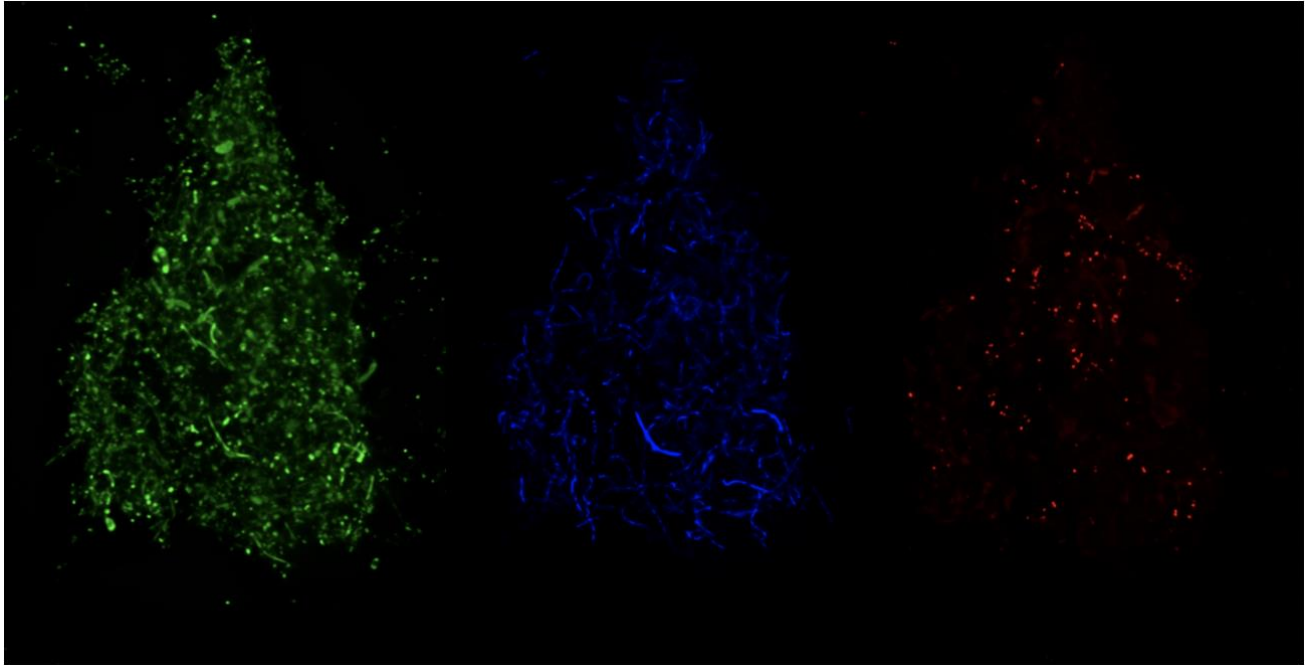


Figure 24a. Media containing *Megasphaera HOT 123* with EUB338 (Alexa 488) probe in green on the left for all eubacteria, FUNU probe for *F. nucleatum* in blue in center and MEGA 123 (Alexa 647) probe for *Megasphaera HOT 123* in red on the right on Ultra Stick slides.

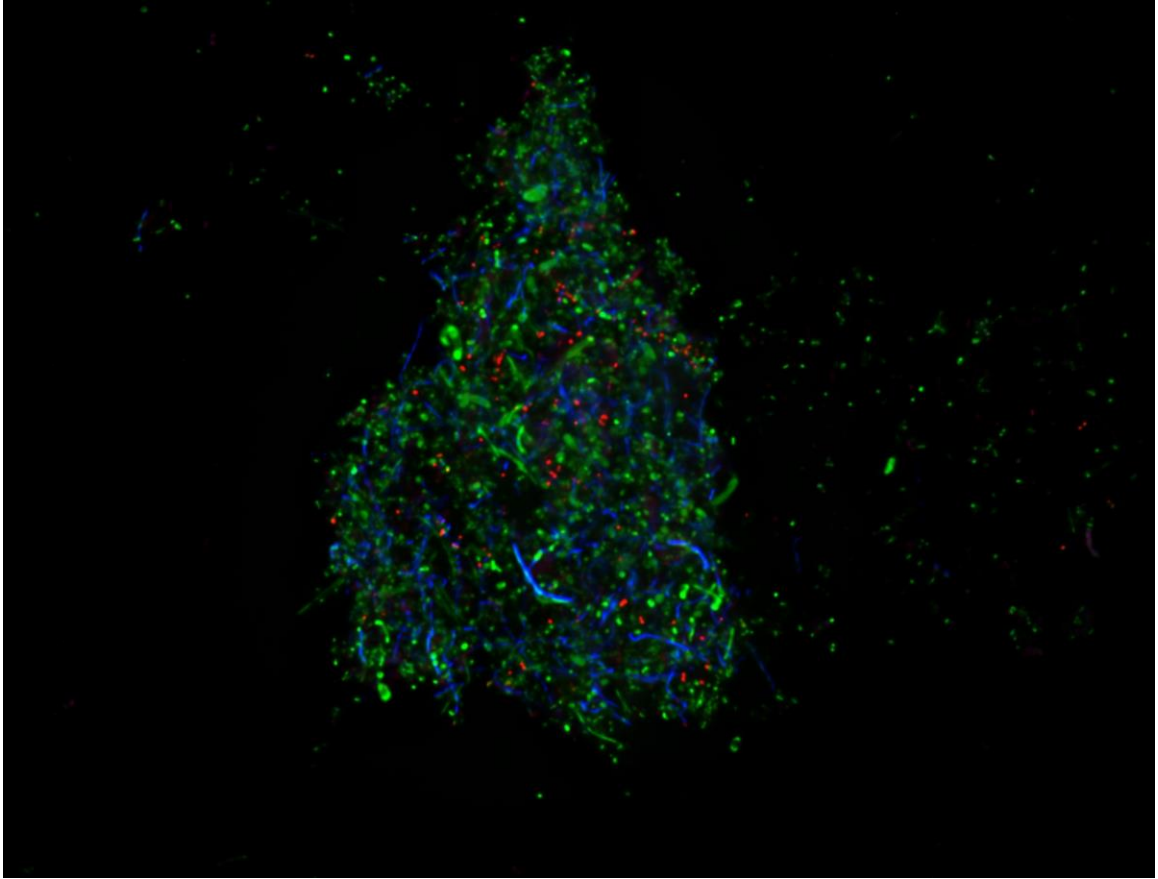


Figure 24b. Media containing *Megasphaera HOT 123* with EUB338 (Alexa 488) probe in green for all eubacteria, FUNU probe for *F. nucleatum* in blue and MEGA 123 (Alexa 647) probe for *Megasphaera HOT 123* in red on Ultra Stick slides.

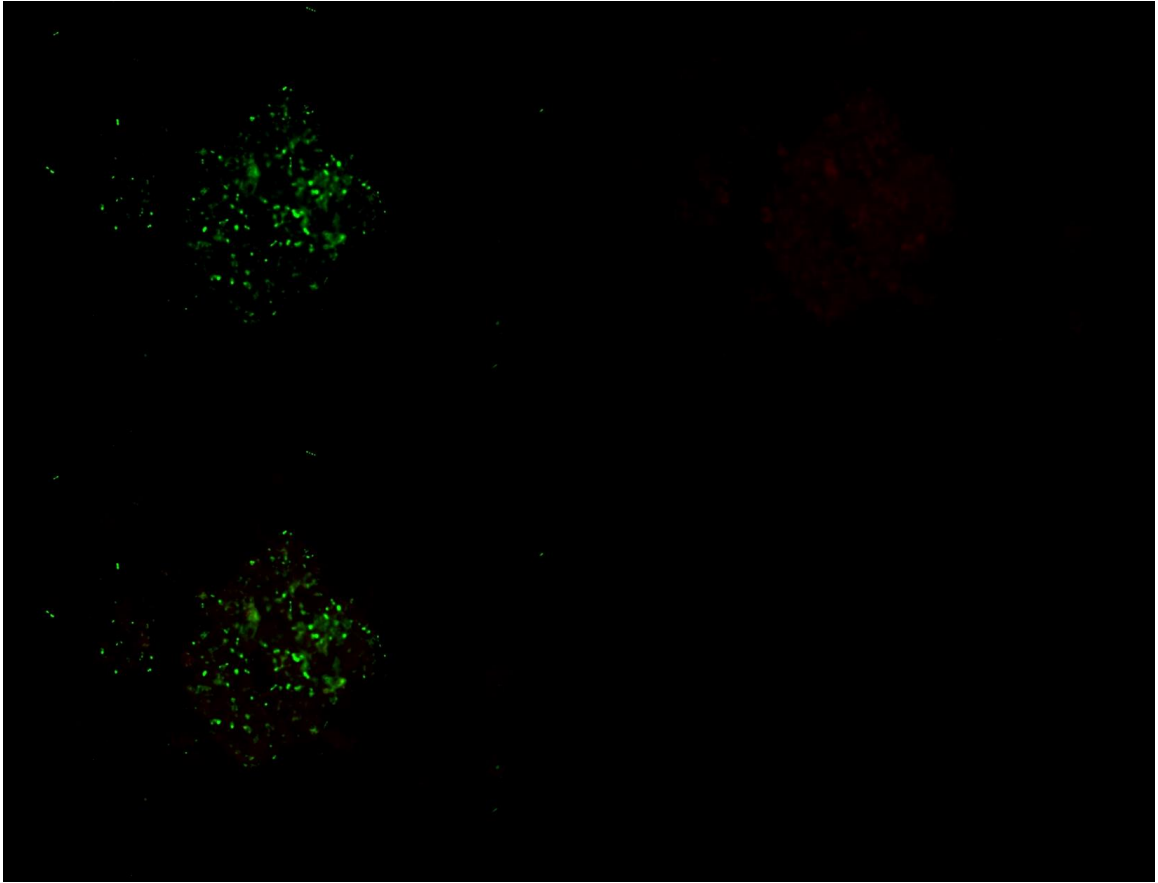


Figure 25. Media without *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red. Top left EUB338 alone, top right MEGA 123 alone and bottom right shows both EUB338 and MEGA 123 probes.

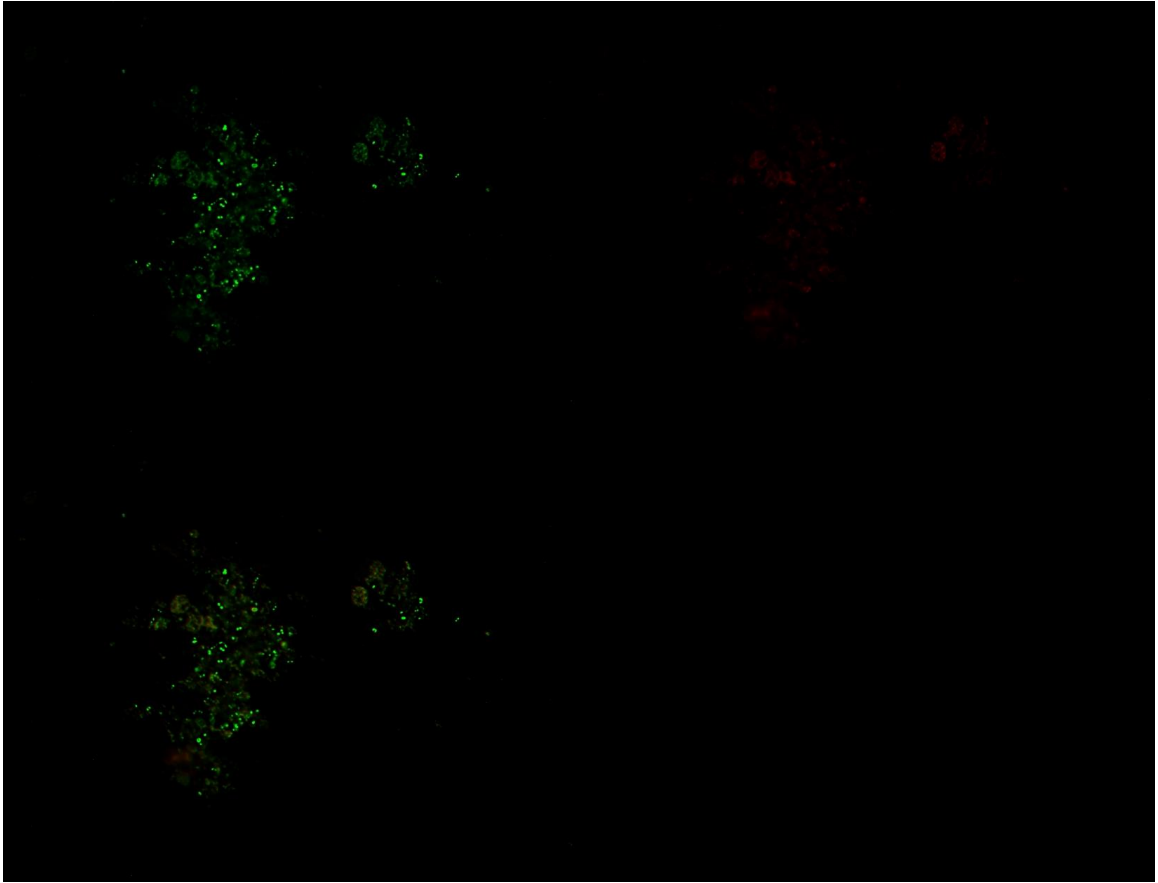


Figure 26. Media without *Megasphaera* HOT 123 with EUB338 (Alexa 488) probe in green for all eubacteria and MEGA 123 (Alexa 647) probe for *Megasphaera* in red. Top left EUB338 alone, top right MEGA 123 alone and bottom right shows both EUB338 and MEGA 123 probes.

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