Tracking *In situ* Biofilm formation in Response to Iron – Using Culture Independent Approaches

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Abstract

Various different biofilm attachment surfaces play a role indicating the type of organisms capable of initial attachment and colonization to form a mature biofilm. Inert surfaces like glass are less selective compared to specific surfaces like metals, however microorganisms are known to colonized various surfaces like metals, medical supplies etc. However, it is unknown how microorganisms preferentially attach to one surface over another in a given steady ecosystem. The main question of this independent hypothesis is do diverse microbial communities respond and change in presence of a desired attachment surface/substrate. This can be answered by tracking the dynamic development of a biofilm community formed in response to a particular substrate using molecular and advance imaging tools.

Introduction

Bacteria present in any given environment are often found adhered to various surfaces, forming biofilms. These surfaces can vary depending on the surrounding environment, for example in the aquatic oligotrophic environment bacteria are transiently attached to insoluble particulate matter whereas in nutrient rich environments like activated sludge, bacteria are attached to flocs. It is the attachment substrate surface, which is of importance in natural as well as the artificial environment that dictates the type and structure of the biofilm. The biofilm structures differ in degree of complexity, ranging from single-cell layered constructions, to well developed multilayered complex biofilm. A free floating bacterial cell initiates the attachment process by adhesion molecules, that allow binding to the selective surface, this attachment can be reversible or irreversible depending on the adhesion molecules produced. Next the attached cell either divides or recruits other bacterial cells via cell-cell communication at a critical cell density, resulting in formation of a microcolony, and eventually as the number of cells increase they produce exopolysaccharides that form a three dimensional biofilm. (Fig.1)
The main goal of this independent project was to study the changes in microbial communities in presences of a given substrate in the natural environment. To study this School Street Marsh was selected as the environmental site and different forms of iron were tested as a substrate for attachment and community analysis.

Material and Methods

To study the effect of biofilm formation on different attachment surfaces a variety of different substrates were introduced in School Street Marsh for two weeks. Four different types of attachment surfaces were used, etched glass slides coated with ferrihydrite slurry, iron and copper foils. These were compared to the control plain etched glass slides. In order to hold test and retrieve samples back for analysis, each individual glass slide or foil was attached to a string. Samples were collected at regular intervals (1day, 1 week and 2 weeks) and prepared either for microscopy and different molecular techniques like *in-situ* hybridization or RFLP analysis.

A] Experiment set up and collection

Material-

Plain etched glass slides, ferrihydrite coated slides, iron and copper foils, binder clips, epoxy glue, string and tape.

Glass slides were first washed with milli-Q water, next with ethanol and etched using Armour etch (glass etching cream).

Ferrihydrite coated glass slides were made by making a ferrihydrite slurry by dissolving 300mM FeCl3 into one liter of milli-Q water, pH was adjusted to circumneutral with NaOH pellets and slurry was washed with milli-Q water several times before using. Approximately 8ml of slurry was pippetted onto an etched glass slide and placed in the 50°C oven for 30 minutes to help coat the glass slide.

Assembly-

Plain etched glass slides, ferrihydrite coated slides, iron and copper foils were attached to a string either by using epoxy glue or a binder clip which was then attached to a string. Slides and foil were placed in between the oxic/anoxic zone of the marsh and held steady by tying the string to the trees above.

Collection-

Slides were collected at the set time interval, and fixed direct either in 3% glutaraldehyde for 2 hours for SEM imaging or in 4%formaldehyde overnight for *in situ* hybridization or in 1X PBS for enrichments or other molecular analysis.

B] Molecular Analysis

DNA Extraction-

DNA was extracted from freshly collected different slides and foils, by washing and scraping the biofilm off using 1XPBS and this solution was used for DNA extraction using the MOBIO Power Soil DNA extraction kit, following the manufactures instructions. Eluted DNA was stored at -20°C for further analysis.

16S PCR Amplification-

Primers:

Archaeal:

Forward- 4F 5'-TCCGGTTGATCCTGCCTG- 3'

Reverse- 1392R 5'-AGGGGCGGTGTCTACA- 3'

Bacterial:

Forward- 8F 5' – AGAGTTTGATCCTGGCTCAG – 3'

Reverse- 1492R 5' – GGTTACCTTGTTACGACTT – 3'

PCR Cycle:

Archaeal	Bacterial
1. 95°C for 5 minutes	1. 95°C for 5 minutes
2. 94°C for 30 second	2. 95°C for 30 second
3. 56°C for 30 second	3. 46°C for 30 second
4. 72°C for 1.5 minutes	4. 72°C for 1.5 minutes
5. Repeat steps 2-4 for 35 cycles	5. Repeat steps 2-4 for 30 cycles
6. 72°C for 6 minutes	6. 72°C for 5 minutes
7. Hold @ 4°C	7. Hold @ 4°C

Fluorescence In Situ Hybridization (FISH)/ Catalyzed Reporter Desposition-Fluorescence In Situ Hybridization (CARD-FISH)-

Materials-

Probes Used for FISH and CARD FISH:

Probe	Sequence
Geo3-A	**CCGCAACACCTAGTACTCATC**
Geo3-B	**CCGCAACACCTAGTTCTCATC**
Geo3-C	**CCGCAACACCTGGTTCTCATC**
EUB I,II,III	GCTGCCTCCCGTAGGAGT GCAGCCACCCGTAGGTGT GCTGCCACCCGTAGGTGT
NON 338	ACTCCTACGGGAGGCAGC
ARCH915	GTGCTCCCCCGCCAATTCCT
Gam42a & comp	GCCTTCCCACATCGTTT GCCTTCCCACTTCGTTT
Alf968	GGTAAGGTTCTGCGCGTT
Beta42a & comp	GCCTTCCCACTTCGTTT GCCTTCCCACATCGTTT
Delta495a & comp	AGTTAGCCGGTGCTTCCT AGTTAGCCGGTGCTTCTT
Delta495b& comp	AGTTAGCCGGCGCTTCCT AGTTAGCCGGCGCTTCKT
Delta495c& comp	AATTAGCCGGTGCTTCCT AATTAGCCGGTGCTTCTT

Sequences in bold are cy3 mono-labeled probes

General FISH/CARD-FISH protocol-

FISH, CARD-FISH and DAPI staining was first optimized on high iron containing sediment and water samples collected from school street marsh, in order to modify Pernthaler et.al., 2002, FISH protocol for performing *in situ* hybridization directly on biofilms developed on glass slides with/without iron oxide. Samples and glass slides were fixed as described above. The sediment samples were centrifuged at 16,000 rpm for 5 minutes, decanted the supernatant and resuspend the pellet in 1X PBS (pH7.6), and repeated this step twice. Resuspend the pellet in a mixture of 1X PBS to ethanol (40:60); this can be stored in -20°C, till further processing. To disperse cells attached to sediment /iron oxide particles, 50µl of the sediment pellet was suspended in 1:1 ratio of 1X PB to ethanol and sonicated. The sonication probe was set at about 4 watts (instrument in Rowe) and the suspended pellet was kept on ice and pulsed 12 times for 30 seconds each (Ishii et. al., 2004 and Dagmar's protocol). 50µl of the sonicated sediment sample was diluted with 2ml of 1X PBS and filter through 0.2µm GTTP filter. Filters were air-dried and then were stained with DAPI to see if these were suitable for CARD-FISH or stored

at -20C till further processing. The following CARD-FISH steps were performed as described in the lab manual.

Fixed glass slides containing biofilms were washed twice in 1X PBS and then stored in 1:1 mixture of 1X PBS to ethanol at -20°C till further processing either with FISH/CARD-FISH. Each fixed slide was cut into seven-eight pieces for hybridization with specific probes.

Sequential FISH/CARD-FISH-

To study dual hybridization on one biofilm glass slide, sequential hybridization was done. First a CARD-FISH was done using general eubacterial (EUB I, II, III) or delta probes and then specific probes were further used to identify individual groups, or a mono labeled Geobacter specific probes were used.

C] Imaging

Scanning electron microscopy-

Specimen Processing:

SEM was used to visualize the three-dimensional surface structure of the biofilm. Slides were fixed as described above, after fixing three serial 20 minutes washes in 1X PBS were done. Next, were series of ethanol dehydration steps in 50%, 70%, 85% and 95% for 10 minutes on ice and then three 15 minute washes in 100% ethanol were done. Fixed specimen slides were dried using the critical point dryer and spatter coated.

Laser Scanning Confocal Microscopy-

Zeiss inverted LSM-510 laser scanning confocal system was used to image FISH/CARD-FISH slides, using 2 photon confocal at 790nm for DAPI and 488nm for FITC labeled probes and 560 for cy3 labeled.

Epifluoresences Microscopy-

CARD-FISH/FISH prepared slides were observed under the GFP filter for FITC and Alexia 488 labeled probes and dsRED was used for cy3 and Alexia 594 labeled probes were used.

Results-

One-day-old biofilms analysis-

Both plain and iron oxide coated glass slides were observed under the SEM, and saw initial attachment by variety of different organisms, also some eukaryotic cells were observed along with a lot of extracellular polysaccharide matrix (Fig. 2 a, b, c, d). DAPI staining was done on these one day slides and approximate thickness was determined by using z-stack thickness, biofilm formed on the plain slide was about 64μm and iron coated slide was about 30μm thick (Fig. 3 a, b).

One-week-old biofilm analysis-

One week old slides under SEM look remarkably different compared to one day old slides, after the initial attachment, it appears that there are microcolony formation on the iron oxide coated glass slide, however very few cells were observed for the plain glass control slides (Fig. 4a, b, c, d). Figure 4e shows an SEM image of a one-week-old biofilm developed on an iron foil; small rod shaped cells can be seen. It was very difficult to image biofilm on the iron foil as it was negatively charged and the particles moved due to the electron bombardment.

An unusual observation was seen when the DAPI stained one-week plain glass slides showed a thick biofilm formation compared to SEM images, indicating loss of attached cells during SEM sample preparation (Fig. 5a, b, c, d). On iron-coated glass slides very distinct rod shaped cells were observed attached only to the iron oxide particles. Biofilm slides were hybridized with general eubacterial, non338 and proteobacterial specific probes, along with an archaeal probes, however no archaeal signals were detected. Thickness of these biofilms was detected by doing a z stacking and then depth profiles were generated using LSM software. The plain slide was about~124μm and the iron coated was ~90μm thick. Example of a CARD-FISH with delta probes was done on one-week-old plain and another one done with delta and Geobacter on a one-week iron oxide glass slide which is shown in figure 6 and 7.

Two-week-old biofilm-

Slides collected after two weeks had a very thick biofilm development, as seen with depth profiling. SEM image of plain glass slide showed excess extracellular matrix as seen in fig.8a. Iron oxide coated glass slide showed an increase in bacterial cells attached to the iron oxide particles along with extracellular matrix production. FISH and CARD/FISH analysis done on two-week samples showed relative abundance of beta, gamma and delta proteobacteria, also showed 164μm thick shown in figure 9. Most of the delta positive cells were also Geobacter positive, indicating colonization of the iron oxide particles essentially by Geobacter species (Fig 10).

Copper foil as s substrate could not be carried out till end as, the one of the foil was lost to the swamp during sample collection and the other was used for DNA extraction and RFLP analysis.

Table 1 shows the approximate numbers of probe specific hybridized cells that were enumerated by counting several fields through the biofilm. This result shows that the iron coated glass slide had a dominant population of Geobacter compared to the plain glass slide, indicating substrate specific attachment preference.

Table1 FISH and CARD/FISH enumerations

	Alfa	Beta	Gamma	Delta	Geobacter
- Iron	++	+++	++++	++	+
+ Iron	+	+/-	+++	+++	++

Molecular Results

DNA was extracted from samples collect after two week analysis using the MOBIO power soil kit, but very low yield of DNA was obtained. Also, 16S PCR amplification was tried to generate clone libraries and RFLP patterns, but due to low DNA yield and presence of iron, no amplified product was obtained.

Discussion and Conclusions

The SEM images showed that the one-day-old biofilm on both the plain and iron oxide coated slides; there was an initial attachment of microorganisms. However, more eukaryotic organisms were observed on the plain glass compared to the iron oxide coated slides. Both had various bacterial morphologies, rod, and cocci along with filamentous bacteria. The biofilm population changed drastically after one week of incubation, the plain glass slides had established a thick three dimensional structure about 124μm where as the iron oxide coated glass side was only about 90μm, based on depth profiles done using the LSM software. Another observation was that in spite of having a thicker biofilm formation on the plain glass slides poor SEM images were obtained, indicating loss of biofilm during SEM sample preparations. A more interesting observation was on the iron oxide coated glass slide, that the cells attached only on the iron particle and not on the glass surface (no cells were observed under SEM) and these were mostly rod shaped, which appear to be dividing, maybe forming a microcolony. The two-week-old biofilm showed more ordered structures on both plain and iron oxide coated glass slides, indicating that dynamic biofilm formation. Relative increase in the number of rod shaped bacteria colonizing the iron oxide particles was observed, along with increase in extracellular matrix around these bacteria.

Comparing the thickness of the biofilm establishment overtime, indicates that the iron oxide coated glass slide is a selective substrate and only specialized organisms like iron reducing bacteria colonized whereas on the plain glass slides everyone can attach and form a biofilm, this observation is further supported by the *in situ* hybridization experiments.

In situ hybridization using HRP conjugated and mono-labeled probes showed the relative abundance of the different groups of proteobacteria present in the biofilm population and also allowed to identify *Geobacter* populations on these biofilms. There were a large number of cells colonizing the plain glass slide; those were mostly the beta proteobacteria or the gamma proterobacteria. Since these slides were placed between the oxic and anoxic zone, I could have enriched for some iron oxidizing bacteria like *Leptothrix* species or *Thiobacillus ferrooxidans* belonging to the beta proteobacterial group. High population of gamma positive cells on both the plain as well as the iron oxide coated

slides could possibly indicate presence of *Shewanella* species associated with iron reduction. Based on *in situ* hybridization using mono-labeled *Geobacter* specific probes, confirmed a relative high abundance of *Geobacter* species on the iron oxide coated slides, also a low signal was detected on the plain glass slides.

Future Experiments

This project has shown some very preliminary results that certain bacterial cells selectively attach to specific substrates like iron oxide in high numbers, but non selectively attach to inert surfaces like glass. One very interesting follow up hypothesis is that what makes iron reducing bacteria like *Geobacter* selectively attach to iron oxide coated glass surface in high numbers compared to plain glass slide in iron rich ecosystem. One interesting question is how does *Geobacter* sense iron, ferrotaxsis? Is it the availability of the iron (form of iron) or requirement of a solid surface to perform co-ordinated functions in a density dependent manner? Other follow-up experiments could be routine questions like who are these other bacteria besides *Geobacter* that are growing on iron oxide? This can be answered by performing 16S identification analysis and setting up enrichments from these biofilms.

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Biofilm formation

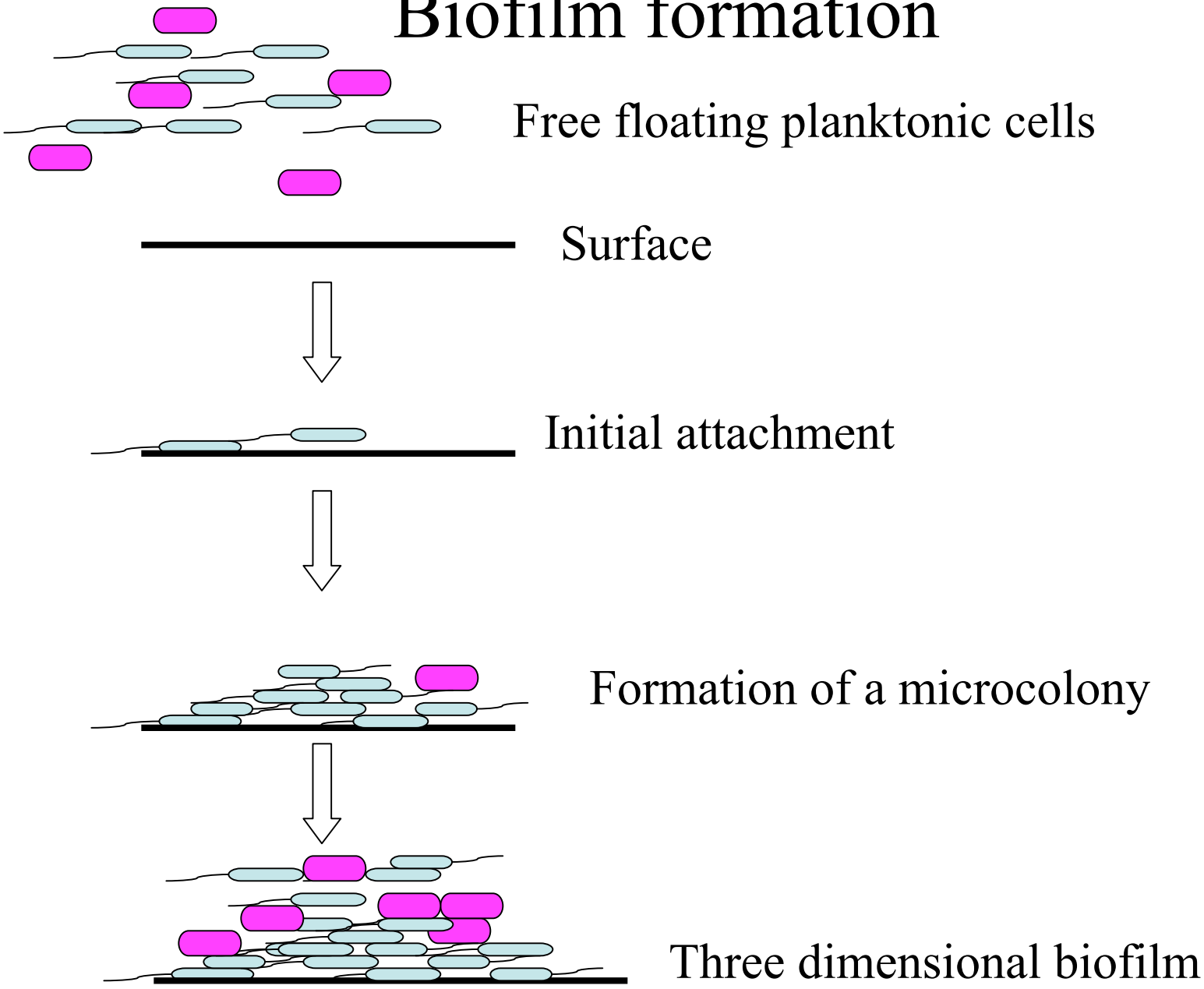
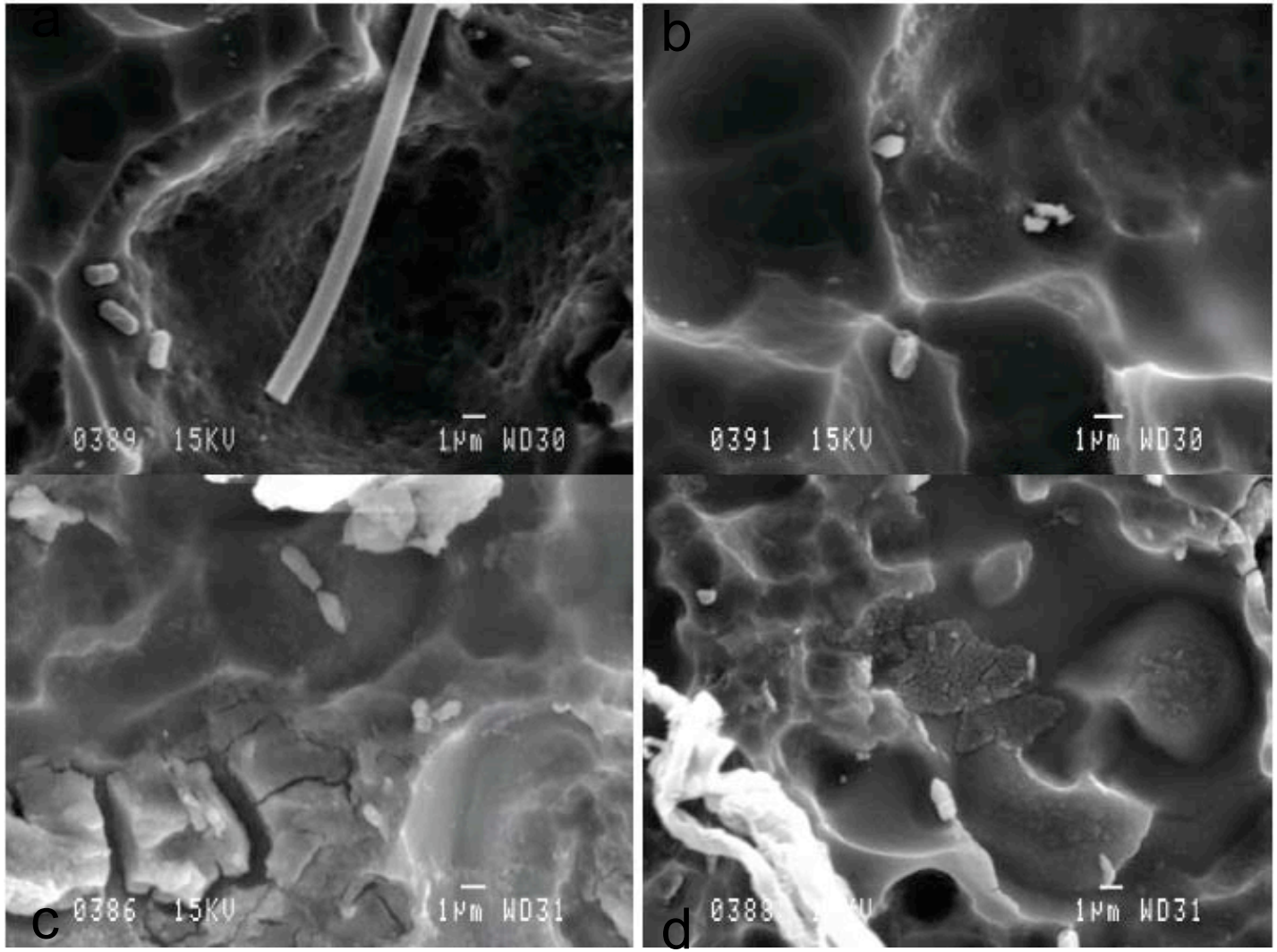


Fig.1

Figure 2

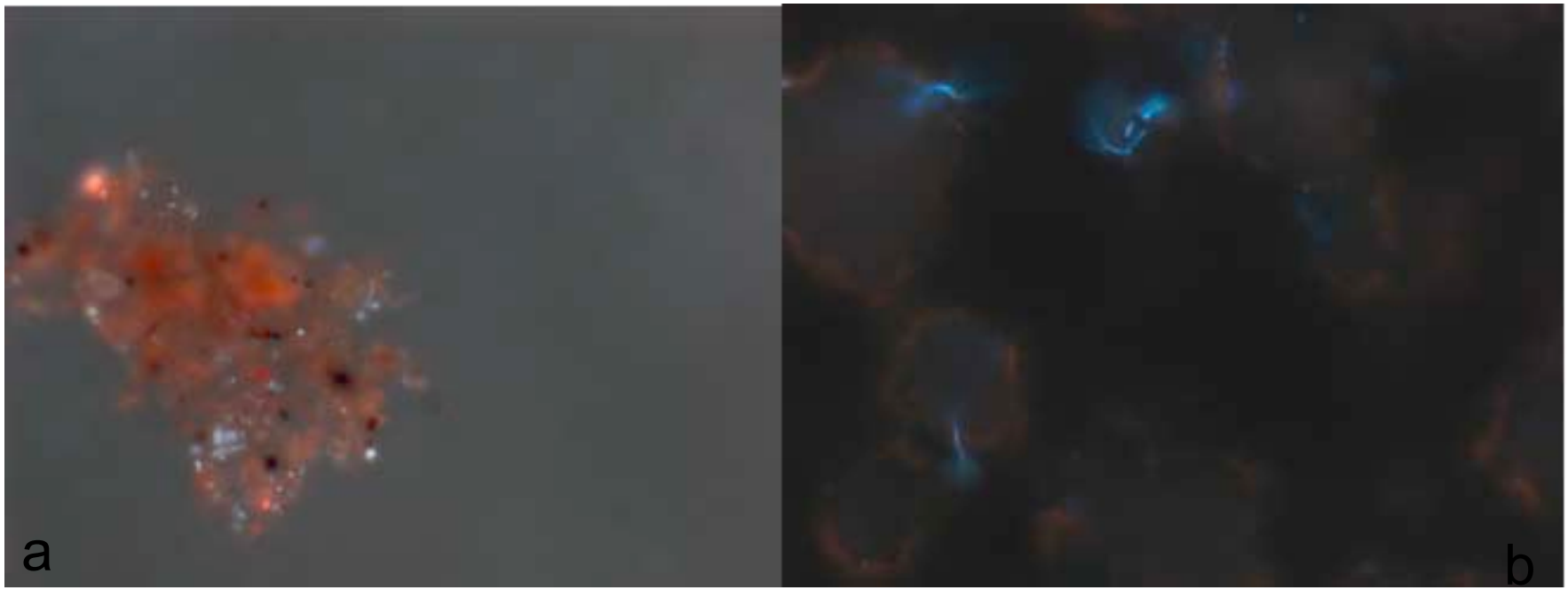
Plain glass slide, one day old



Iron coated one day old slides

Figure 3

One day DAPI Stain

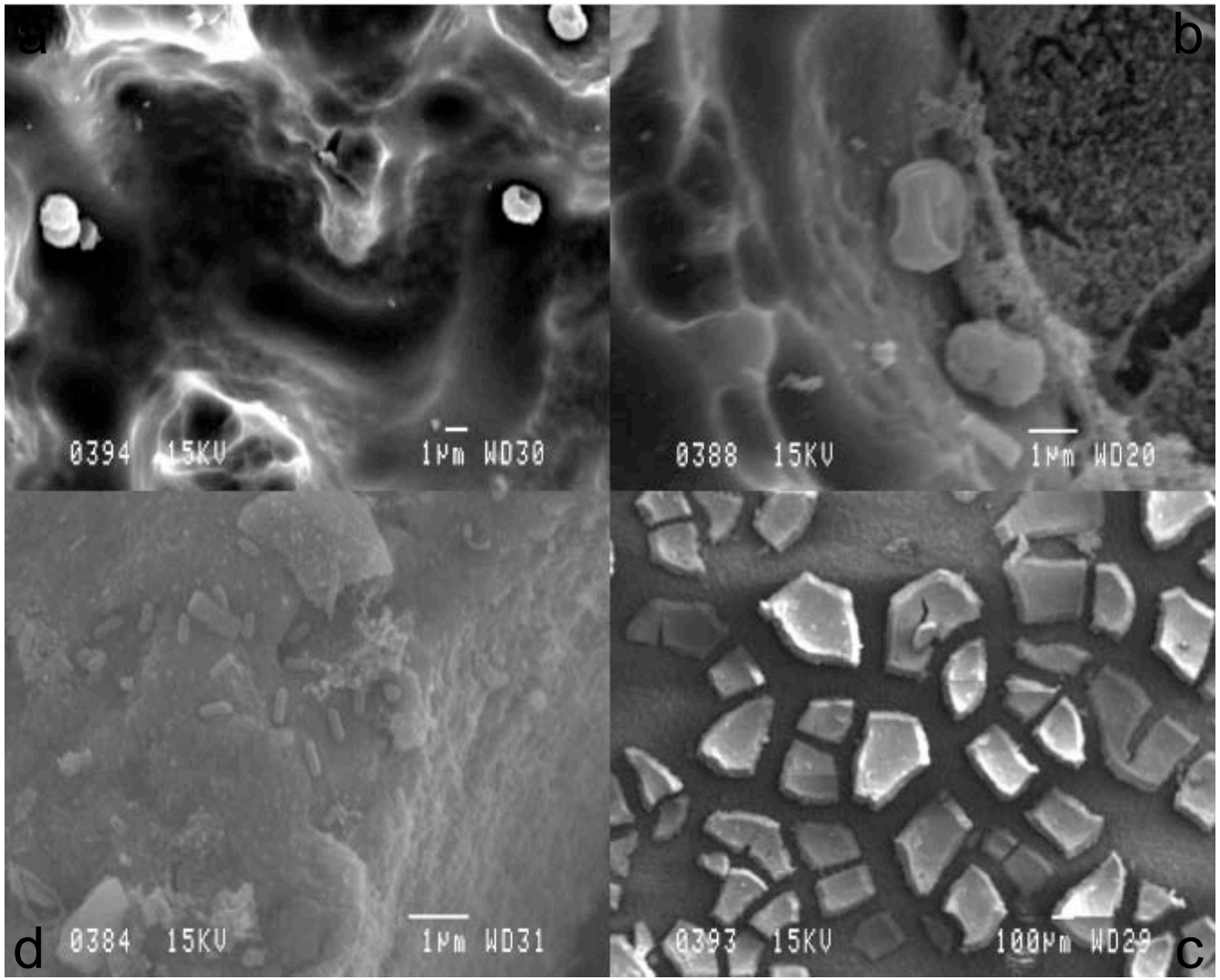


Plain

Iron coated

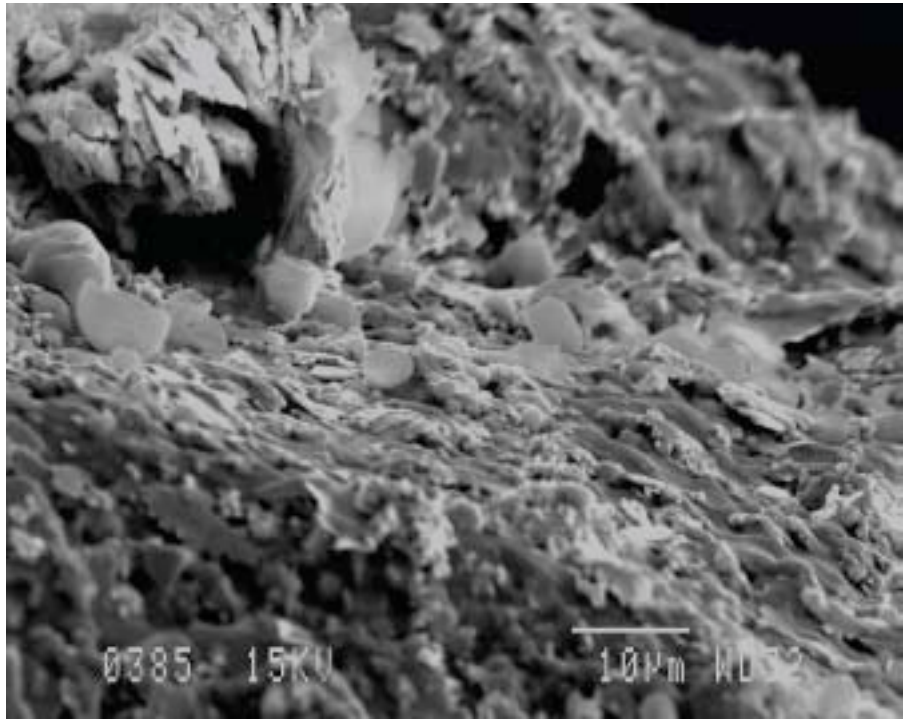
Figure 4

Plain One Week



Iron coated One Week

Figure 4e



Iron Foil One week

Figure 5

DAPI Stain for Depth Profiles

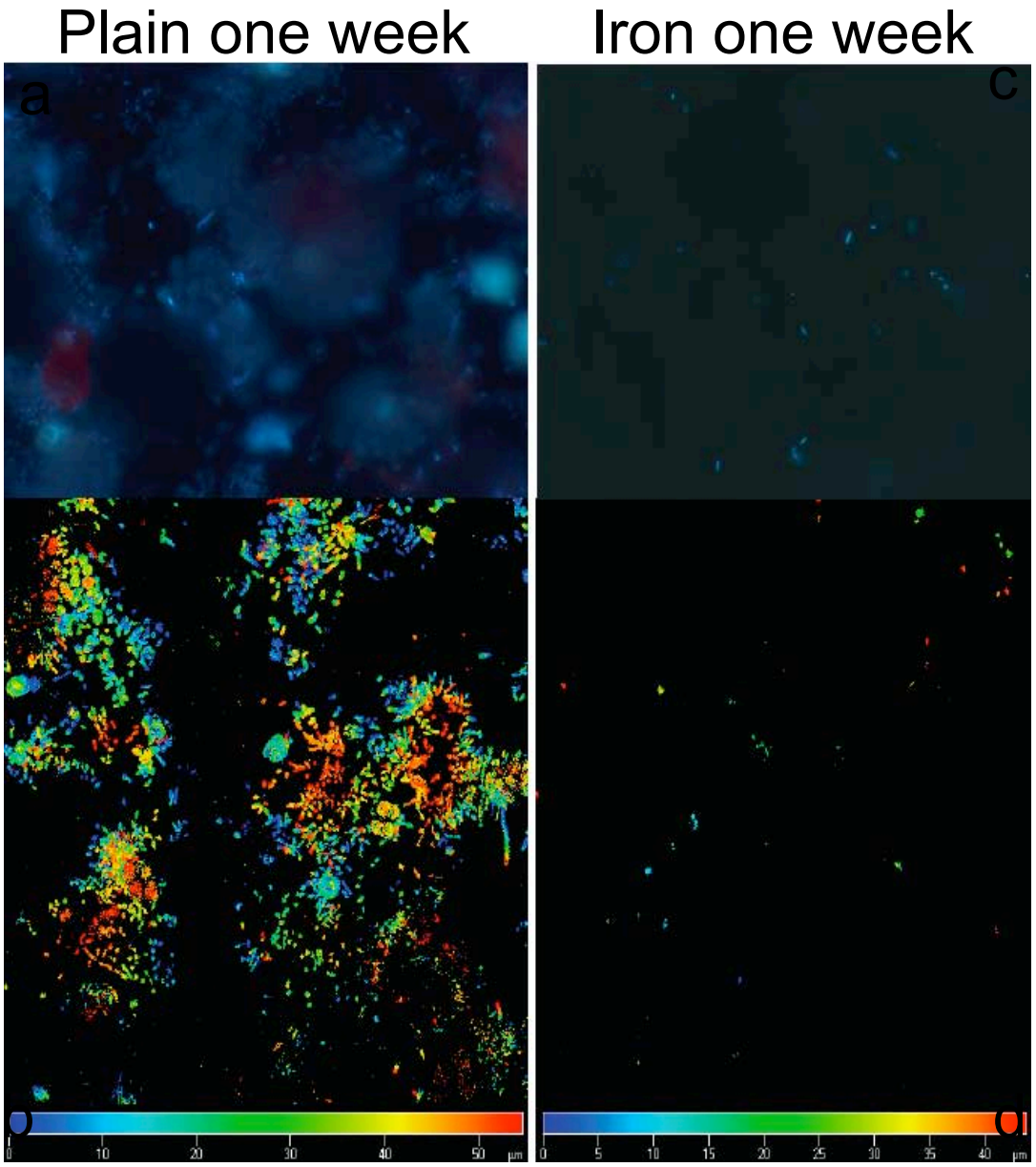
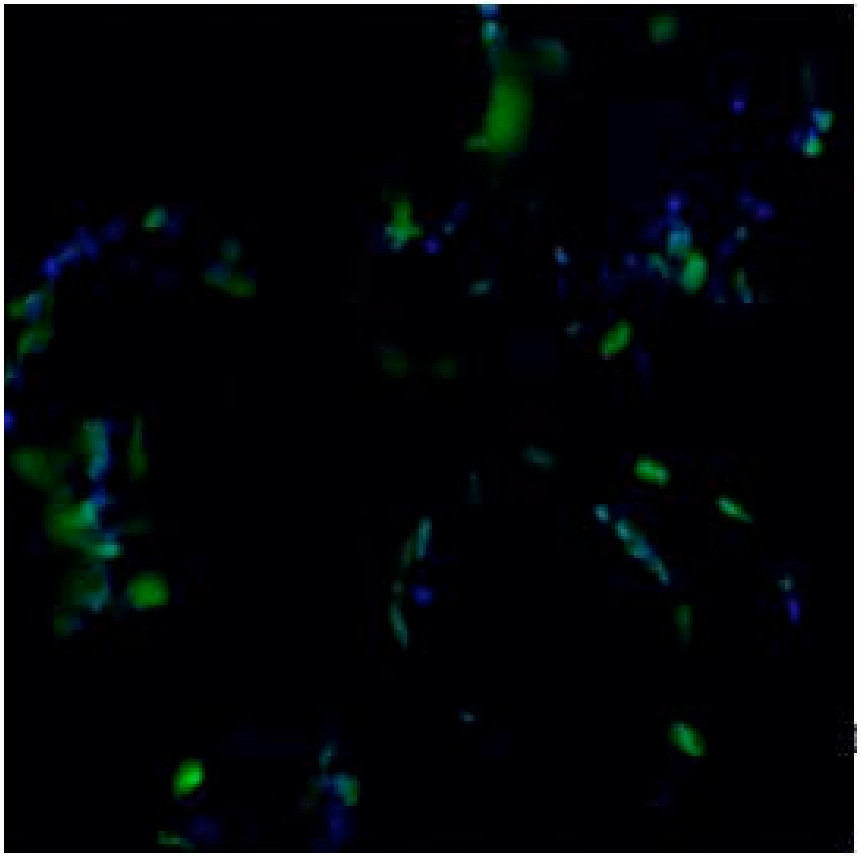


Figure 6

One week iron oxide slide

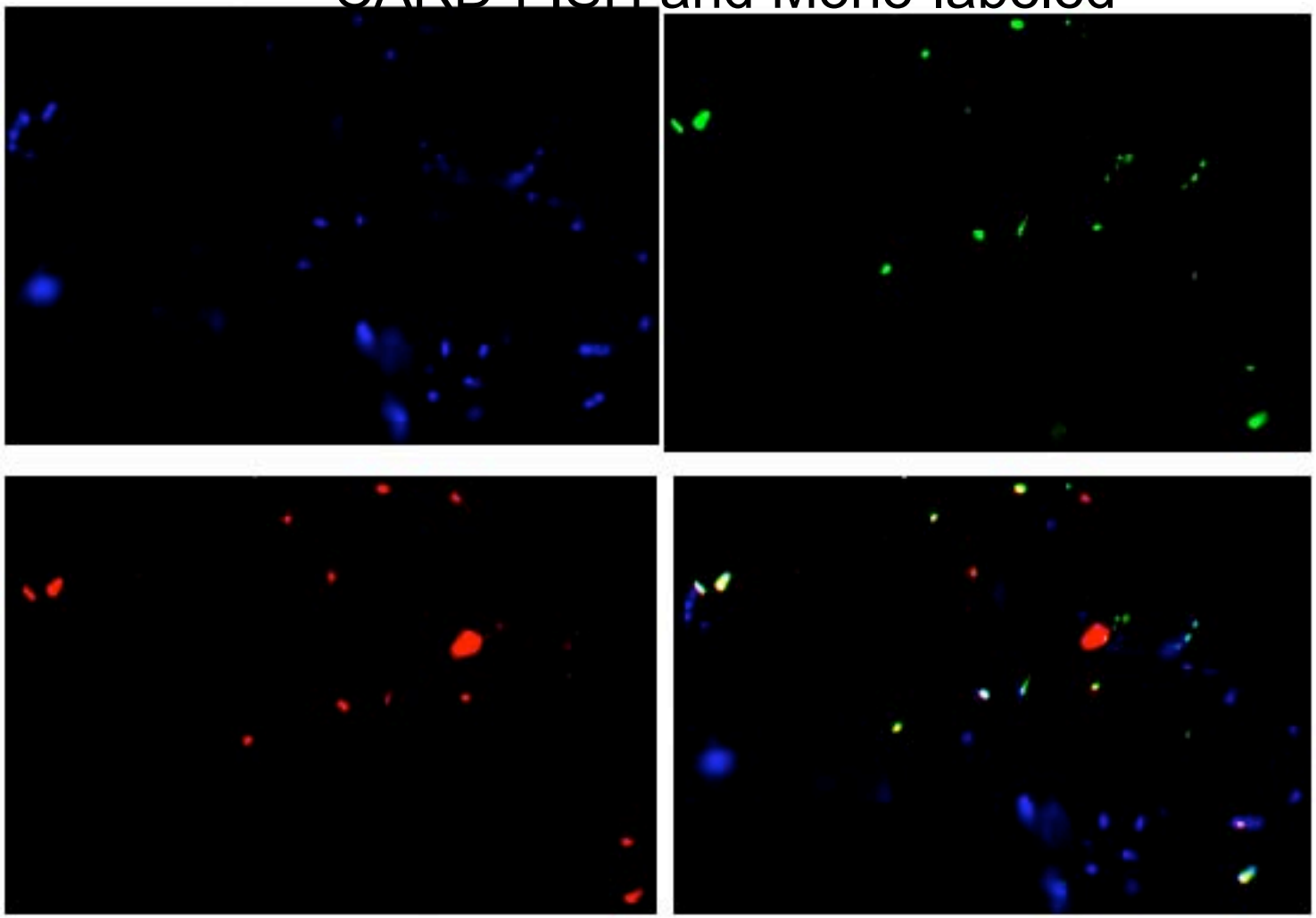


-DAPI
-Delta specific

Figure 7

One week iron oxide slide

CARD-FISH and Mono-labeled



-DAPI

-Delta

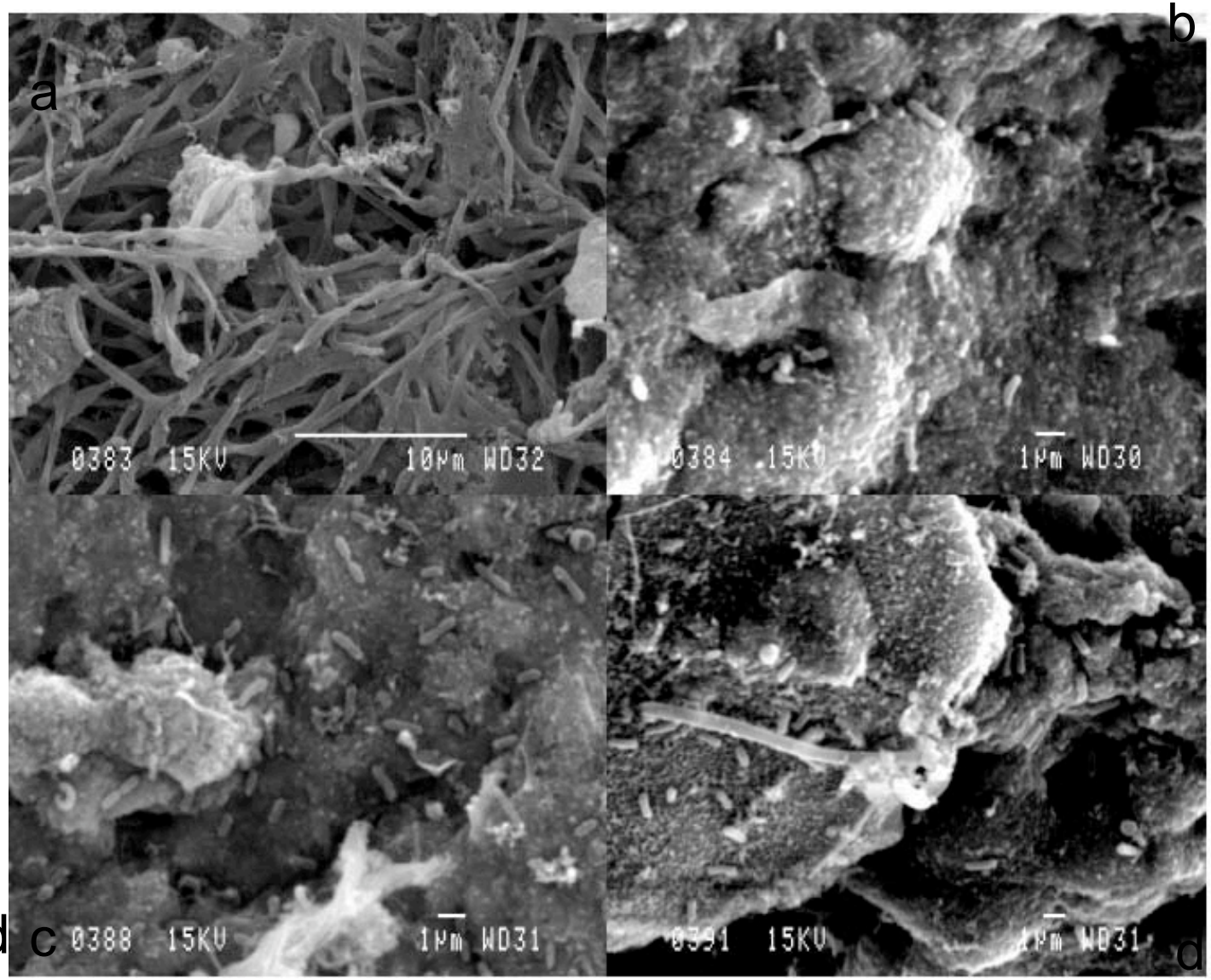
-Geobacter

Overlay

Figure 8

Two week SEM

Plain



Iron Coated

Iron coated

Figure 9

Two week iron oxide biofilm

Depth Profile

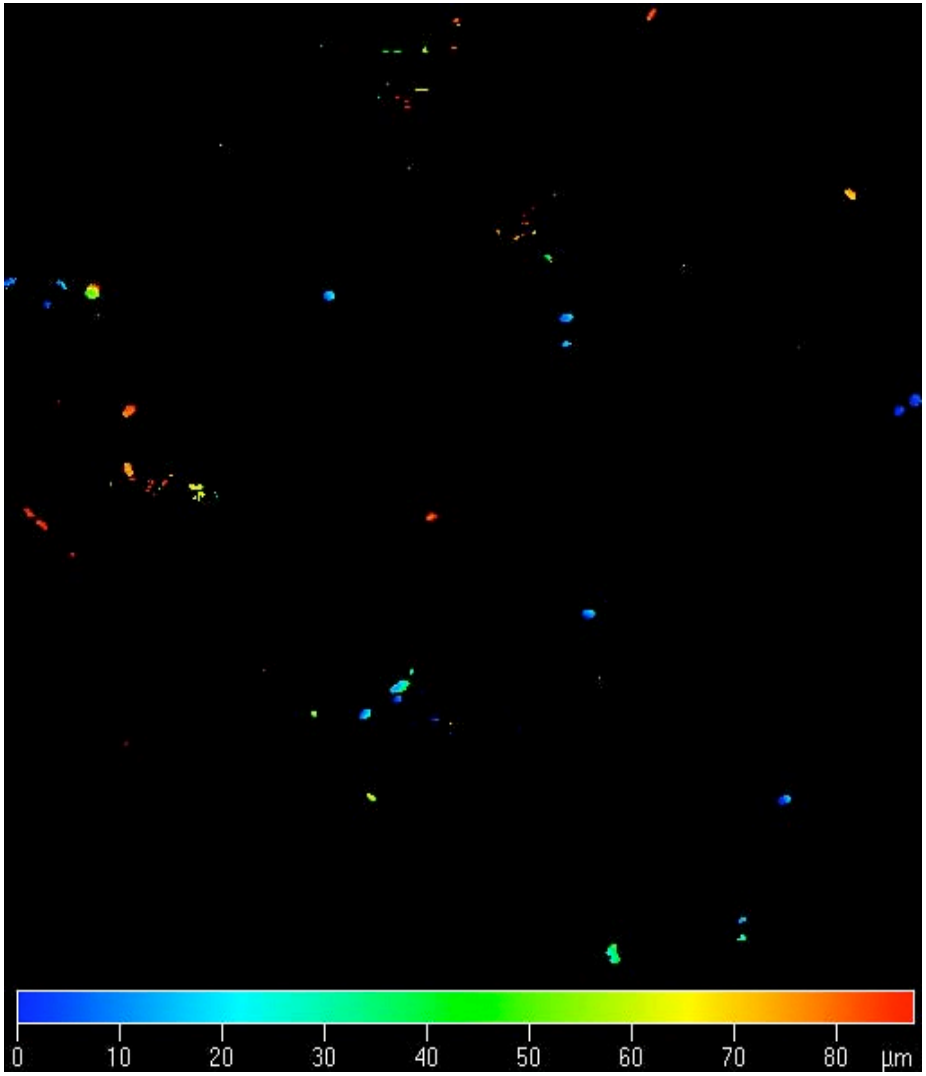


Figure 10

Mono-labeled FISH on two week iron oxide