Comparison of PFA Fixation Time VS Fluorescent Signal Intensity in Model Organism and Identification of Growth Curve of Human Oral Microbiome Community Member

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Abstract

Imaging bacteria using FISH requires fixation of cells to preserve their natural state. The method of fixation used, and the fixation period, play a role in fluorescent signal intensity. Using a model organism E. coli K12, fixation in 4% PFA for a period of 1.5 hours and 4 days were imaged using a LSCM (Laser Scanning Confocal Microscope). The fluorescent signal intensity of the images were compared via visual inspection, a qualitative measure. Fixation of *E. coli* K12 in PFA for 1.5 hours resulted in a stronger signal intensity compared to the 4-day fix. In addition, S. mutans' (human oral microbiome member) growth curve was identified for future fixation comparison.

Introduction

Imaging fluorescently labeled bacterial cells occurs in a step wise fashion. Cells are first fixed for preservation of natural state and then subsequently permeabilized by a chemical agent (E.g. Organic solvents: ethanol, acetone, methanol etc., or detergents such as the enzyme saponin or Triton X-100 and others.) [3]. The permeabilized cells can then be hybridized with a fluorescently labeled oligonucleotide probe corresponding to the complementary rRNA sequence of the target, then washed to remove excess probe [1]. Single cells can be subsequently identified via LSM (laser scanning microscopy) [1]. Fixation represents a vital step in acquiring a robust image and optimizing signal intensity of bacterial cells, but fixation presents various challenges. The type of fixative as well as the period of fixation play a role in signal intensity. There are two general classes of fixatives: (1) Cross-linking fixatives; (paraformaldehyde) PFA, formalin, glutaraldehyde, etc. Cross-linking fixatives contain aldehydes that create covalent bonds between proteins thus preserving natural cell structure. (2) Precipitating fixatives; EtOH, MeOH, acetone etc. Precipitating fixatives act by denaturing proteins via disruption of hydrophobic interactions [2]. 4% PFA was used in this investigation. The reaction with PFA (i.e. formaldehyde) with proteins is characterized by the combination of nitrogen (on amino acids such as lysine) with formaldehyde allowing for the subsequent formation of methylene bridges (that increase with time) between protein side chains. Therefore, the time at which a bacterial cell fixes in PFA may affect the degree to which the probe can penetrate the cell membrane, which might inhibit the probes ability to locate and anneal to its corresponding rRNA sequence. Testing this on a model organism *E. coli* K12 (whose growth curve is well defined) represents the first step in a progression towards investigating community members of the oral microbiome, and eventually yield insight on the diverse community structure.

S. mutans (ATCC 25175) represents a good model organism of the human oral microbiome. Most initial colonizers of the pellicle (rich in salivary and membrane-bound exoproteins) are in the streptococci genus (and *Actinomyces spp.*), which are capable of adhering to the tooth surface (after brushing) and to other bacterial community members [5]. In addition, *S. mutans* can develop into a cariogenic biofilm in response to a sugar rich (especially from sucrose consumption) environment characterized by high levels of acidity (4). Optimization of signal intensity of *S. mutans* represents a useful tool in analysis of oral microbial films because of its association with the tooth surface, diverse interactions within the community, and role in tooth decay. To achieve this mid-log phase (corresponding to peak production of ribosomal rRNA, and thereby maximized probes per cell) of *S. mutans* needs to be identified by creation of growth curve.

Methods

Growth Curve and Fixation:

Inoculated cell culture tube containing 5ml, 1x Luria Broth (LB) Media (Difco™ BD, cat no. 244520) with *E. coli* K12 from glycerol stock for overnight incubation at 36 °C. From the overnight culture 20uL was transferred to LB Agar Plate and incubated overnight. A single bacterial colony was chosen from the plate and incubated overnight in 5ml LB. The following day four culture tubes were created and inoculated with 50uL of the second overnight culture. One of the culture tubes was dedicated for growth curve measurement at 600nm using an Eppendorf Biophotometer (cat no. Hamburg 22331) (fig.1). The other three were retrieved for fixations at 120, 156, and 246 minutes respectively for fixation in mid-log range of growth. The cell cultures were then fixed in 4% PFA for 4 days. The fixed cultures were subsequently centrifuged and resuspended in 500uL of 1x PBS (ThermoFisher, Cat no. 10010049) ensuring complete resuspension and then 500uL of pure 200 proof ethanol was added in addition. The fixed cells were then stored at -20 °C for two weeks. This process was conducted in the same way for comparison with 4% PFA fixation for 1.5 hours. With growth periods analogous to prior description.

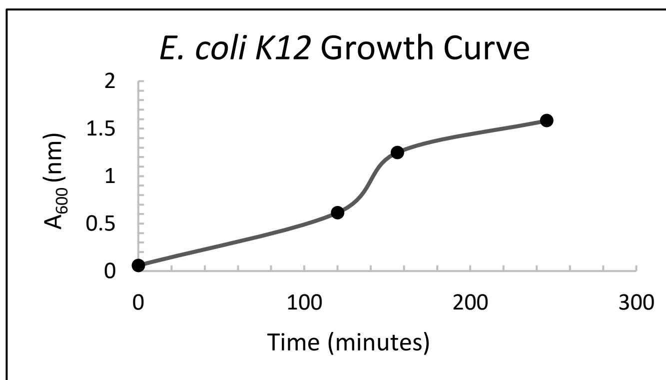

Figure 1: E. coli K12 growth curve measured by absorbance (600 nm) vs time (mins)

Labeling and Mounting for FISH Imaging:

The fixed *E. coli* cells were then labeled for fixation. From each of the time points a 10uL aliquot was retrieved and hybridized for a period of 18 hours in 90uL FISH buffer (*.9 M NaCl, .02 M Tris, .01% SDS, 20% HiDi Formamide, 2 M Rhodamine-Red X fluorescent label)*. Cells were then washed twice (Wash 1: (*.9 M NaCl, .02 M Tris, .01% SDS, 20% HiDi Formamide);* Wash 2: *(.9 M NaCl, .02 M Tris, .01% SDS)*. 40uL of from each time point were mounted to Thermofisher Ultrastick™ adhesion slides and placed in a humid chamber for a period of one hour. The slides were then dehydrated via series of ethyl alcohol of

Time (hours)	A_{600} (nm) Tube 1	A_{600} (nm) Tube 2	A_{600} (nm) Tube 3	A_{600} (nm) Tube 4	A_{600} (nm) Tube 5	Average A_{600} (nm)
0	0.13	0.042	0.039	0.03	0.05	0.0582
9.5	0.237	0.283	0.29	0.317	0.4	0.3054
10	0.287	0.328	0.358	0.42	0.527	0.384
11	0.577	0.644	0.707	0.711	0.927	0.7132
13	1.51	1.576	1.674	1.743	1.809	1.6624
14	1.785	1.73	1.879	1.785	1.725	1.7808
15	1.743	1.723	1.714	1.752	1.723	1.731
16	1.645	1.623	1.635	1.673	1.657	1.6466
17	1.573	1.582	1.574	1.599	1.625	1.5906

Table 1: Time in hours of growth for 5 replicates of S. mutans and corresponding absorbance at 600nm. The average of these values was taken and plotted with standard error to visualize the growth curve.

increasing concentrations (50%, 75%, and 100%, pure 200 proof EtOH) for ten seconds per increasing concentration and allowed to dry for ten minutes. The coverslips were adhered with Invitrogen Prolong Gold™ antifade mountant and left to dry for two days. Images were then obtained using Laser Scanning Confocal Microscopy (Zeiss). For optimal detection of fluorescent signal, the detector was set to capture wavelengths of ≥561 nm. Images were captured at 40x magnifications, power level 10, and a gain of 625. Images were then processed by Zeiss image rendering software and compared via visual inspection. 1.5 hour and 4-day fixation in 4% PFA were labeled and mounted identically.

Identification of *S. mutans* growth curve:

Inoculated cell culture tube containing 5ml of 1x Brain Heart Infusion (BHI) Media (Difco™ BD, Cat no. 211059) with *S. mutans* (ATCC25175) from glycerol stock for overnight incubation (36 °C). From the overnight culture 20uL was transferred to BHI agar plate and allowed to incubate for two days. Colonies were observed after two days and the plate was stored at 4 °C. for two days. A single colony was then chosen from the plate and a second cell culture tube contain 1x BHI Media and allowed to incubate for 32 hours. After 32 hours, 100ulx5 was placed in to a new set of 5x5ml of 1x BHI Media for

incubation. Initial absorbance of each of the five tubes was measured, and subsequently repeated at 9.5, 10, 11, 13, 14, 15, 16, and 17 hours. The average absorbance of each timepoint was then plotted with standard error.

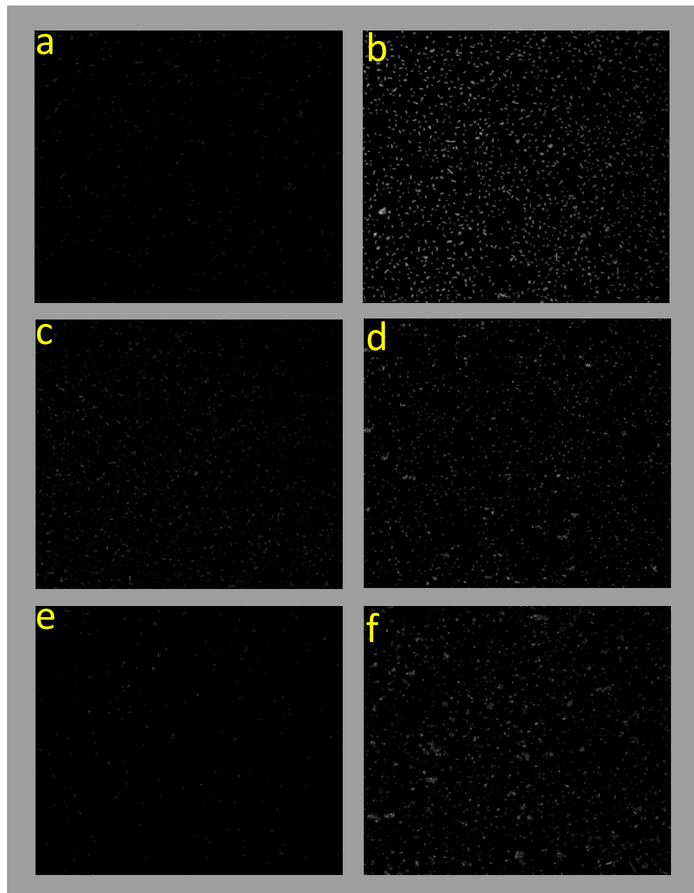

Figure 3: A side by side comparison of Fluorescent signal vs Fixation time for model organism E. coli K12. (a,c,e): 2-hour, 3-hour, and 4-hour growth points fixed in 4% PFA for 4 days respectively. (b,d,f): 2-hour, 3-hour, and 4-hour growth points fixed in 4% PFA for 1.5 hours respectively.

Results

Comparison of FISH signal Intensity:

FISH signal intensity was compared side by side via visual inspection (fig. 2). Visual analysis indicated that fixation in 4% PFA for four days yielded a significantly decreased fluorescent signal when compared to the shorter-term fixation (1.5 hours). Short-term fixation yielded the highest signal intensity at the two-hour growth point (fig.2b), seeming to consistently drop as the time of growth increased (fig.2d,2f). The longer-term fixation (4 days) yielded the highest signal intensity corresponding to the three-hour growth point (fig. 2c), but all growth points (2,3, and 4 hours) exhibited a poor signal overall.

Identification of *S. mutans'* growth curve:

S. mutans' growth curve was plotted using the data from table 1. Extrapolating from the plot (fig. 3) lag phase was identified to be from approximately 0 to 9.5 hours. Log phase was from 9.5-13 hours, with mid-log phase occurring 11-13 hours. Stationary phase appeared to begin around 13.5-14.5 hours, after which decrease of absorbance was observed likely related to cell death.

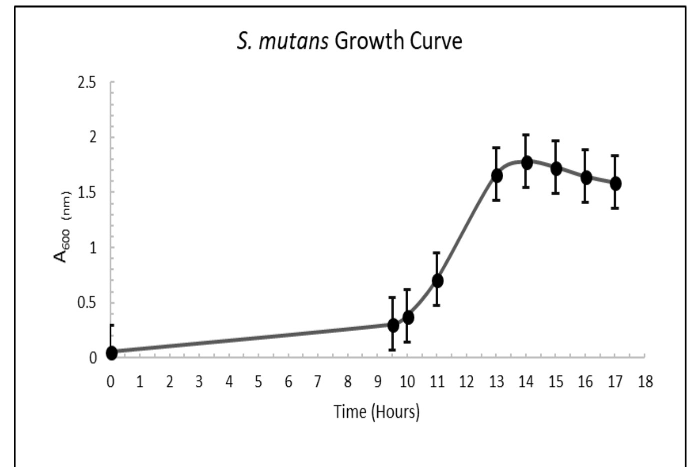

Figure 2: Growth curve of S. mutans measured by absorbance (600nm) vs time (hours), with vertical bards representing standard error. Note: Time point for 13 hours was omitted due to faulty transcription.

Discussion

Fixation time in the cross-linking fixative PFA clearly seems to affect the signal intensity. This may be due to a higher extent of polymerization as time proceeds. This could prevent the oligonucleotide probe from penetrating the cell membrane and finding the ribosome which it labels. This warrants the testing for an array of different fixation times for comparison of signal intensity. It may be that an even shorter fixation period could result in a more robust fluorescent signal. Now that the growth curve of *S. mutans* has been clearly identified, an array of fixation methods may also be compared for this target organism. In the future, a comparison of FISH signal intensity VS (fixation type and time) will be conducted on *S. mutans.* Image analysis would be conducted using ImageJ for a quantitative analysis of fluorescent signal intensity. Types of fixatives being considered are: Carnoy's Solution (10:30:60, glacial acetic acid: chloroform: ethanol), methanol, 10% formalin, 2% PFA, and 4% PFA. Understanding the effect of fixation methods on fluorescent signal intensity will help to obtaining more robust images and thereby allow

for better identification of community structure within the human oral microbiome.

References

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