

NUCLEAR MAGNETIC RESONANCE MICROSCOPY OF
STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

By

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April 2004

TABLE OF CONTENTS

1. INTRODUCTION	1
Biofilms.....	1
<i>Staphylococcus epidermidis</i>	2
Nuclear Magnetic Resonance	2
Fundamental Concepts.....	3
Net Magnetization.....	4
Magnetization Dynamics and the Rotating Frame.....	4
Excitation	6
Relaxation	7
The NMR Signal.....	8
Imaging	9
Gradients.....	9
Spin Echoes.....	10
k-space	11
Slice Selection.....	11
2-D Imaging.....	12
Encoding for Motion.....	13
Propagators	14
Thesis Goal	15
2. MATERIALS AND METHODS.....	16
Bacterium.....	16
Growth Medium.....	16
Flow Cells	17
Biofilm Growth Setup.....	17
Structural Images and Fluid Flow Data	17
Time Lapse Imaging	18
NMR Imaging System	19
NMR Experiment Setup.....	20
T ₁ Weighted Images.....	21
T ₂ Maps.....	22
Making T ₂ Maps	23
Velocity Maps.....	24
Making Velocity Maps	25
Propagators	25
3. RESULTS AND DISCUSSION.....	27
Clean Capillary	27

TABLE OF CONTENTS – CONTINUED

Theoretical Solution for Flow in a Square Capillary	27
NMR Measurements for Flow in a Clean Capillary	28
Biofilm Fouled Capillaries.....	30
Structural Images of Biofilms.....	30
Copper Sulfate Contrast Agent	30
Magnevist Contrast Agent	32
Velocity Maps Around Biofilms	34
Time Lapsed Imaging	44
Propagators	46
4. CONCLUSIONS.....	49
LITERATURE CITED	51
APPENDICES	54
APPENDIX A: SCANNING ELECTRON MICROSCOPY IMAGES.....	55
Introduction.....	56
Procedure	56
Results and Discussion	57
APPENDIX B: NMR SPECTROSCOPY OF BIOFILMS.....	59
Introduction.....	60
Procedure	60
Results and Discussion	61
APPENDIX C: 3 MM CAPILLARY	63
Introduction.....	64
Procedure	64
Results and Discussion	64
APPENDIX D: CTC DYE EXPERIMENTS	66
Introduction.....	67
Procedure	67
Results and Discussion	68

LIST OF FIGURES

Figure	Page
2.1. Setup for Growing Biofilms in Flow Cells.....	18
2.2. Setup for NMR Imaging System	20
2.3. Pulse Program for T_1 Weighted Images	21
2.4. T_2 Map Pulse Program.....	22
2.5. Making a T_2 Map	23
2.6. Pulse Program for Velocity Encoding	24
2.7. Making a Velocity Map	25
2.8. Propagator Pulse Program	26
3.1. MatLab Solution for Flow in a Square Capillary	28
3.2. NMR Velocity Maps in a Clean Capillary	29
3.3. Comparison of Measured and Theoretical Velocity Data	29
3.4. NMR Images with Copper Sulfate Contrast Agent	31
3.5. NMR Images of Biofilm Structure	32
3.6. Biofilm Sloughing	34
3.7. Velocity Maps of a Biofilm Fouled Capillary	35
3.8. Slice Orientation for Figures 3.9-3.13	36
3.9. Paravision Images of Wall 1 and Other Side.....	37
3.10. MatLab Plots of Slice Wall 1.....	38
3.11. MatLab Plots Through the Middle of the Capillary	39

LIST OF FIGURES - CONTINUED

3.12. MatLab Plots of the Slice at Wall 2.....	40
3.13. MatLab Plots of Slice Against Other Wall.....	41
3.14. T_2 Versus z-direction Velocity for Wall 1	42
3.15. Y Versus X-direction Velocity Components.....	43
3.16. Time Lapsed Structural Images	44
3.17. Time Lapsed Velocity Maps During Biofilm Growth.....	45
3.18. Propagator Data Comparison With $\Delta=15\text{ms}$	47
A.1. SEM Image	57
A.2. Resolutions of SEM.....	58
A.3. NMR Spectroscopy at 250 MHz.....	61
A.4. NMR Spectroscopy at 500 MHz.....	62
A.5. Clean 3mm Capillary	65
A.6. Biofilm Fouled 3mm Capillary.....	65
A.7. CTC Dye Experiments.....	68

ABSTRACT

Nuclear Magnetic Resonance (NMR) microscopy is well-suited for investigating living systems since it is innocuous and non-invasive. In addition to imaging internal structures of systems, NMR microscopy techniques can be used to obtain information about transport phenomena, such as fluid velocities and diffusion. The goal of this research was to determine the applicability of NMR imaging techniques to studying transport properties in biofilms. *Staphylococcus epidermidis* biofilms were investigated because of their importance in medical implant infections. NMR experiments were used to image biofilm structure and the fluid flow patterns in one millimeter square glass capillaries. Results showing the heterogeneous structure of biofilms are congruent with confocal laser microscopy images. The advantage of using the NMR techniques to image biofilm structures is that there are no light or laser penetration barriers and the innermost regions of the structures can be easily revealed. In addition to imaging the biofilms, velocity distributions have been mapped for the one millimeter capillary system. Laminar flows in clean, square capillaries display axial velocities that are both uniform and symmetrical while non-axial components of velocity are not present. In contrast, a biofilm fouled capillary displays irregular flow patterns in the axial direction along with distinct non-axial secondary flow perturbations. These results demonstrate that biofilms impact bulk flow in ways that cannot be ignored when modeling their impact on transport in bioreactors and medical devices. This work establishes NMR microscopy as an important biofilm research tool which can spatially resolve structural characteristics and transport processes in biofilm fouled systems.

INTRODUCTION

Biofilms

Biofilms are multi-cellular communities of microorganisms which are attached to surfaces and encased in a polysaccharide matrix known as EPS. They are encountered nearly everywhere with some being a mere nuisance, like toilet bowl scum, while others are involved in life threatening infections in cystic fibrosis patients [1]. Internal structures of biofilms are impossible to see with ordinary light microscopes and the major tool for imaging them is the confocal laser microscope [2]. Additional techniques to corroborate the confocal images and elucidate new information about biofilm systems are always welcome.

NMR techniques are innocuous and non-invasive which makes them an excellent tool for studying biofilms. Another major advantage of NMR is its ability to probe any depth in the biofilm structure, a common limitation of other microscopy methods due to light and laser attenuation. NMR imaging techniques can be used to image a biofilm structure, map velocity distributions and characterize the diffusion in biofilm systems [3].

High-resolution NMR images of biofilms have recently been published in the literature [4, 5]. In addition, NMR has proven itself as an effective tool for characterizing the diffusion in various biofilm systems [6] including microbial mats [7], agar plate grown *Pseudomonas aeruginosa* [8], and methanogenic granular sludge [9, 10]. NMR imaging of fluid flow in and around biofilms has been accomplished with flat-plate biofilm reactors [11, 12], round tube reactors [4], and square capillaries [5]. Since no

other imaging technique is able to investigate these opaque systems, NMR imaging has also been employed to study biofilm systems in porous media [13, 14] and packed bed reactors [15, 16, 17].

Staphylococcus epidermidis

The bacterium *Staphylococcus epidermidis* is a biofilm forming species that is normal flora on humans, usually found on the skin and mucous membranes [18]. Infections can occur in humans and, although rarely life threatening, can persist for long periods of time because of the tenacity of biofilms [1]. *S. epidermidis* was chosen for these experiments due to its clinical relevance and because it forms thick biofilms of a few hundred microns which are visible with the naked eye in glass capillaries. In addition to being prolific, *S. epidermidis* biofilms grow relatively quickly reaching the desired thickness of a few hundred microns in two to three days.

Nuclear Magnetic Resonance

The following discussion is a brief overview of the quantum mechanics behind NMR. If a more rigorous understanding is desired the texts by Abragam [19] and Slichter [20] provide the fundamental quantum mechanical theory of NMR. Some additional helpful NMR references are: Callaghan [21], Codd [22], Farrar [23], Farrar and Becker [24], Fukushima and Roeder [25], and Hornak [26].

Fundamental Concepts

The spin quantum number, I , is equal to an integer or half-integer for a given particle. For any isolated nucleus, the possible energy levels are dependent on I and equal to $2I+1$. The energy separation between the energy levels is given by

$$\Delta E = \mu B_0 / I \quad (1.1)$$

where μ is the magnetic moment and B_0 is the magnetic field which the nucleus is experiencing. The magnetic moment is defined as:

$$\mu = \gamma \hbar I / 2\pi \quad (1.2)$$

where γ is the gyromagnetic ratio, which is constant for a specific nucleus, and $\hbar$ is Planck's constant. This small nuclear magnetic moment is what allows nuclei to interact with large, static magnetic fields, like a compass interacting with the earth's magnetic field.

The frequency of radiation that a particle can absorb when placed in a static magnetic field is derived from the Bohr relation and given by

$$\omega_0 = \gamma B_0 \quad (1.3)$$

where ω_0 is frequency of radiation or the Larmor frequency, γ is the gyromagnetic ratio and B_0 is the magnetic field strength. When this exact frequency of energy is absorbed by the particle it is able to transition between the energy states and it said to resonate at that frequency. Equation (1.3) is the fundamental equation of nuclear magnetic resonance and shall be derived later using a classical mechanics approach.

In addition to magnetic moment, another important property that nuclei possess is an angular momentum represented by

$$J = \hbar I \quad (1.4)$$

where $\hbar$ is Planck's constant divided by 2π .

Net Magnetization

A proton, with $I = \frac{1}{2}$, possesses two possible energy levels. When placed in a uniform, constant magnetic field, the magnetic moments of an ensemble of protons will align parallel with the magnetic field in a lower energy state, or anti-parallel with the field at a higher state. The equilibrium distribution of the protons, in accord with the Boltzmann distribution, is given by

$$N_+/N_- = \exp(-\Delta E/kT) \quad (1.5)$$

Where N_+ and N_- are the populations of protons in the upper and lower energy states, k is the Boltzmann constant, ΔE is defined above in equation (1.1), and T is the temperature in Kelvins.

From equation (1.5) it can be seen that a small fraction of protons preferentially align with the magnetic field, in the lower energy state. This produces a net magnetization vector, M , in the direction of the magnetic field. This net magnetization can then be treated as a single magnetic moment so that each spin will not need to be considered separately. A classical mechanics approach can then be adopted instead of the quantum mechanical one which has been used thus far [21].

Magnetization Dynamics and the Rotating Frame

The movement of the net magnetization vector in a magnetic field is governed by the classical laws of motion which state that the time rate of change of the angular

momentum, J from equation (1.4), is equal to the cross product of the magnetization vector, M , and the magnetic field it is experiencing, B . Mathematically written

$$dJ/dt = M \times B \quad (1.6)$$

By combining equations (1.2) and (1.4) the relationship between angular momentum and the magnetic moment is given by

$$J = \gamma \mu \quad (1.7)$$

Then, by performing the suitable averaging, one may substitute the net magnetization vector, M , for the magnetic moment of a single proton, μ , and plug into (1.6) [21]

$$dM/dt = \gamma(M \times B) \quad (1.8)$$

Solving (1.8) for a static field, B_0 , along the z-axis

$$M_x(t) = M_x(0)\cos(\gamma B_0 t) + M_y(0)\sin(\gamma B_0 t) \quad (1.9)$$

$$M_y(t) = M_y(0)\cos(\gamma B_0 t) - M_x(0)\sin(\gamma B_0 t) \quad (1.10)$$

$$M_z(t) = M_z(0) \quad (1.11)$$

From equation (1.11) it is demonstrated that the z-component of the net magnetization does not vary with time. However, it does precesses about B_0 at the Larmor frequency derived quantum mechanically as equation (1.3)

$$\omega_0 = \gamma B_0 \quad (1.12)$$

In order to excite the spins, or achieve resonance, another magnetic field, B_1 , oscillating at ω_0 must be applied in the plane perpendicular to B_0 (the transverse plane) [21]. The total magnetic field can then be described as the sum of B_0 , the static field, and B_1 , a time varying field. Since B_0 is constant and the precession about it likewise, it is convenient to introduce a new coordinate system which rotates around B_0 at the

frequency ω_0 . This new coordinate system is called the rotating frame with axes that are denoted x' , y' and z' . The rotating frame simplifies the equation of motion from:

$$dM/dt = M \times \gamma(B_0 + B_1(t)) \quad (1.13)$$

to

$$dM/dt = M \times \gamma B'_1(t) \quad (1.14)$$

so that M only precesses about B_1 at a frequency:

$$\omega_1 = \gamma B_1 \quad (1.15)$$

Excitation

In order to “excite” spins, or make them absorb energy, a short pulse of radio frequency (rf) energy at the Larmor frequency, ω_0 , is applied. M will then precess about B_1 through an angle given by:

$$\theta = \gamma B_1 t \quad (1.16)$$

where θ is the flip angle and t is the length of time the pulse is applied. A flip angle of 90° will produce a magnetization completely in the transverse plane while 180° will move the magnetization to the $-z'$ -direction. Excitation occurs when M is rotated into the transverse plane. The axis which the pulse is applied along is also used to characterize the rf pulse. A pulse applied around the x' -axis will flip the magnetization so that it points along the y' -axis. The notation used for rf pulses is designated as the flip angle subscripted with the axis so that a 90° pulse along the x' -axis would be written: 90°_x . This notation will be used in the pulse sequences given in the Methods section. Signal manipulation can be achieved by cycling the phases of the rf pulses.

Relaxation

When an rf pulse is applied thermal energy is absorbed by the spins in the system and they are said to be ‘excited’. After the pulse, the system begins returning to the thermal equilibrium governed by the Boltzman distribution. This process is known as the spin-lattice relaxation. The spins in the system exchange energy with their surrounding thermal reservoir, or lattice, bringing the longitudinal magnetization, M_z , back to its equilibrium state, M_0 . The time rate of change of M_z is dependent on the spin-lattice or longitudinal relaxation time constant, T_1 , and M_0 . The following equation describes spin-lattice relaxation:

$$M_z = M_z(0)\exp(-t/T_1) + M_0[1 - \exp(-t/T_1)] \quad (1.17)$$

In addition to T_1 there is another time constant called the spin-spin or transverse relaxation time, denoted T_2 . T_2 relaxation is the means by which the transverse magnetization is brought back to equilibrium (zero) through the incoherent exchange of energy between spins [19, 21]. The phase coherence between spins is responsible for transverse magnetization and dephasing of spins is what allows the system to return to equilibrium. The equation relating T_2 to the transverse magnetization is:

$$M_{x,y} = M_{x,y}(0)\exp(-t/T_2) \quad (1.18)$$

A true T_2 refers only to the molecular interactions that cause the transverse magnetization to return to zero. In reality, small variations in the static magnetic field, B_0 , also contribute to the decay of $M_{x,y}$. The time constant which accounts for both of these phenomena is T_2^* where

$$1/T_2^* = 1/T_2 + 1/T_{2\text{inhom}} \quad (1.19)$$

It is difficult to record true T_2 values in imaging experiments since the values measured in imaging experiments are impacted by the switching of magnetic field gradients [21].

The Bloch equations [29] are a set of differential equations which describe the behavior of the magnetization vector in the rotating frame and are given by:

$$dM_z/dt = \gamma M_y(B_0 - \omega/\gamma) - M_x/T_2 \quad (1.20)$$

$$dM_y/dt = \gamma M_x B_1 - \gamma M_x(B_0 - \omega/\gamma) - M_y/T_2 \quad (1.21)$$

$$dM_x/dt = \gamma M_y B_1 - (M_x - M_0)/T_1 \quad (1.22)$$

where ω is the frequency of the rotating frame. These equations describe the motion of the different components of the magnetization vector as it varies with time and hold for all conditions.

The NMR Signal

The spins in a sample are excited when the net magnetization vector, M , is rotated into the transverse plane and precesses. In the transverse plane, M is rotating at the frequency that the spins are emitting which induces a current in the coil that was used to deliver the rf pulse. The detected signal is then processed to output the vectors M_x and M_y which are the real and imaginary data, respectively since quadrature detection is used [21]. The real and imaginary data is then added to give the magnitude signal, called the Free Induction Decay (FID). The FID is a time dependent voltage response signal which decays with time constant T_2 . At any point in time the FID contains the frequencies of all the different protons in the sample. A Fourier Transform (FT) operation is then needed to convert from the time domain to the frequency domain [27]. The FT relationship to

convert between frequency and time domains and *vice versa* is mathematically given by [21]:

$$F(k) = \int f(t)e^{-ikt} dt \quad (1.23)$$

$$F(t) = \int f(k)e^{+ikt} dk \quad (1.24)$$

Where k is the frequency domain, t is the time domain, $f(t$ or $k)$ is the function being converted to $F(k$ or $t)$. The FT of the time signal basically pulls out all the different frequencies that are present. For example, if there is only one frequency present, a pure sample of one component, then the FT will produce a delta function.

Signal averaging is used to distinguish between actual NMR signal and background thermal noise or to improve the signal to noise ratio. As many different signals are acquired and averaged together, the NMR signal will add coherently while the noise will add randomly. The ratio improves as the square root of the number of averages taken. The only restriction to signal averaging is that between signal acquisitions a time of several T_1 's must be allowed to elapse so that the sample is fully relaxed to optimize signal and care must be taken so that no components of earlier excitations exist when averaging faster.

Imaging

Gradients

Magnetic field gradients are simply additional magnetic fields which vary in space and are produced by specially designed coils that are separate from and added to the static field, B_0 . One of their uses in NMR experiments is to determine the location of

spins in a sample. Previously it was seen that the frequency of a proton is proportional to the magnetic field it is experiencing (1.12). So if a magnetic field gradient is applied, which varies with space, then the frequency that the proton emits will be proportional to that gradient and therefore also dependent on space. Shown mathematically as:

$$\omega(x) = \gamma B_0 + \gamma G \cdot x \quad (1.23)$$

where ω is now the space dependent frequency, γ is the gyromagnetic ratio and G is the component of the magnetic field gradient which has been added to the static field, B_0 .

Spin Echoes

Spin echo signals are used in imaging to gain all the spatial information possible out of a sample. If an rf pulse is applied, then a gradient turned on and the FID recorded there is a small portion of important information missing from the very beginning of the signal. This is due to the fact that the coil which produces the rf pulse is also the coil which receives the signal so there is a small dead time needed for it to switch over [25]. Spin echoes allow access to the important initial information by using a 180° pulse to refocus signal at a later time when the signal can be easily acquired.

Spin echoes make use of the fact that the dephasing of spins due to background gradients is a reversible process. This means that if a 180° pulse is applied at a time τ after the 90° pulse, the effects of any background gradients are reversed and at a time 2τ the initial signal is recovered. Sampling this spin echo is equivalent to sampling the original free induction decay.

k-Space

Spin echoes are commonly used for collecting an NMR signal for imaging techniques. What is now needed is a way to sample the signal from spins in a bunch of different volume elements in the object which is to be imaged. This is done through the sampling of the spatial frequency domain or k-space [28], with units of inverse meters, and then converting to spatial domain, in meters, by the Fourier Transform. The detected signal amplitude for a volume element is:

$$S(t) = \iiint \rho(r) e^{2\pi i \mathbf{G} \cdot \mathbf{r} t} d\mathbf{r} \quad (1.24)$$

where $\rho(r)$ is the density of spins in the volume element r , G is the gradient strength, and t is time [21]. The definition of k , the reciprocal space vector, is:

$$\mathbf{k} = (1/2\pi)\gamma \mathbf{G} t \quad (1.26)$$

Substituting (1.24) into (1.23), the FT relationship becomes obvious. From 1.24 it can be seen that k-space is sampled by either varying $\mathbf{G}$ or time. K-space is sampled by moving through time during signal acquisition. At successive time intervals the NMR signal is collected in the presence of a constant gradient. The time between successive intervals is τ_s and the number of samples is N_s .

Slice Selection

Often in NMR imaging we are only interested in a certain area of sample so it would be convenient if we could only image that portion. In order to excite only some spins in a sample a shaped rf pulse is used with a linear gradient. By using a sinc function shaped pulse, only spins in the range of frequencies spatially determined by the

gradient amplitude are excited [21]. The range is determined by the bandwidth of the sinc function:

$$\Delta\omega = 4\pi/t \quad (1.27)$$

where t is the duration of the main lobe. Since the pulse is being applied in the presence of a gradient the frequencies of the spins are spatially encoded for. So, by only exciting spins at a certain range of frequencies a slice of sample is being selected. The gradient strength and direction are used to change the width and orientation of the slice.

2-D Imaging

Two-dimensional imaging of a slice is done through the use of three different gradients. One gradient, called the slice gradient, is turned on during the slice selection process as described above. Another gradient, called the read gradient, is used to encode for the different frequencies in the sample due to the linear dependence of the frequencies on the gradient. Since frequency encoding during signal acquisition can only be used for one direction another encoding, called phase encoding, must be employed for spatial resolution in a second direction. The phase that a spin acquires in the presence of a gradient is:

$$\phi(\tau) = \int B(t,x,y,z)dt \quad (1.28)$$

where the integral is taken from zero to τ and B is the magnetic field magnitude at the point (x, y, z) at time t . From (1.28) it can be seen that if B is spatially dependent it will introduce a spatial dependence on the net magnetization phase.

The easiest way to describe two-dimensional imaging is to move from real space to k -space and switch from our (x, y) coordinates to (k_x, k_y) . When gathering information

in k-space all the points in a 2-D k-space matrix need to be sampled so that it can be Fourier Transformed to produce an artifact free image. If just one point in k-space is missing from the acquisition the image will be ruined since a frequency will be missing from every voxel. Remember from (1.26) that k is linearly dependent on the gradient and the time it is applied. The k-space matrix rows are filled from frequency encoding or the read direction gradient G_x . All the points in a single row are sampled after a single excitation for a single value of k_y . So, all that is left to be done is sample through all the values of k_y , by incrementing G_y and filling up all the rows in the matrix. This is done by running a series of experiments at different values of phase encode gradient, G_y , as shown in the Material and Methods section (Chapter 2).

Encoding for Motion

On top of imaging a sample, NMR allows for the detection of the movement of spins in a sample. This allows for the detection of diffusion and velocities without disrupting the sample in any way. Motion encoding is accomplished by laying another pair of gradients on top of a standard imaging sequence. The two paired gradients are identical in strength and duration with a separation time between them of Δ , one is applied before and one after the 180°_x pulse that forms the echo. This application of a bipolar magnetic gradient field pulse pair for motion encoding is called a Pulsed Gradient Spin Echo (PGSE) [21] experiment.

From equation (1.28) it was shown that the phase is proportional to the magnetic field. So, if a spin has changed position in the time Δ it will experience a different magnetic field, during the second gradient pulse, and the spin will incur a net phase shift

due to its motion. This phase shift is directly proportional to the distance the spin has moved. This displacement, calculated from the phase shift, can then be divided by time, Δ , to get velocity and the amplitude of the signal measured is related to diffusion due to signal decay caused by random motion between the gradient pulse pair.

Propagators

The propagator is the probability that particle, or spin, has moved a distance Δr in a time Δ [21]. Also known as the van Hove self-correlation function [21], the propagator can be used to determine self-diffusion coefficients and pore size in porous media samples. The relationship between the propagator and the collected NMR signal is given by:

$$E(q, \Delta) = \int P(\Delta r, \Delta) e^{(i2\pi q \cdot \Delta r)} d\Delta r \quad (1.29)$$

Where $E(q, \Delta)$ is the NMR signal, q is the area under the gradient ($q = \gamma g \delta / 2\pi$), P is the propagator function, and Δ the observation time. By comparison with equations (1.23) and (1.24) it can be seen that the propagator is the Fourier transform of the acquired NMR signal. For this project, the average propagator was employed as a way to display how every spin within a sample has moved within the observation time Δ , thus providing statistical characterization of the system motion. The average propagator is collected by sampling the entire range of q values, the area under the gradient pulse, and then Fourier transforming the signal to produce the propagator function.

Thesis Goal

The goal of this thesis is to investigate the applications of NMR imaging techniques to researching biofilms. Specifically, NMR will be used to image biofilm structure and the flow patterns within a biofilm fouled capillary.

MATERIALS AND METHODS

Bacterium

The bacterial species used for all the experiments presented in this thesis was *Staphylococcus epidermidis* strain 35984. This particular species was used because it is known to grow thick biofilms in a short amount of time in the laboratory. Thick biofilms were needed for these initial NMR experiments so that we could verify that we were seeing actual biofilm rather than artifacts of the technique. The best resolution of the system is around 5-10 microns, a scale below which molecular motions blur standard NMR imaging methods. Biofilms of a few hundreds microns thick provide a good test of the ability of NMR to image structure. Biofilms of this species are also being studied by other researchers in the laboratory using confocal laser microscopy, which is expected to provide complementary information to that afforded by NMR.

Growth Medium

The *S. epidermidis* biofilms were grown on a standard tryptic soy broth media (DIFCO, Beckton Dickson and Company). Inoculation samples were grown up overnight in medium prepared from 30 grams of broth powder per liter of distilled water, which is the typical preparation technique as given for the media by the manufacturer. For flow cell experiments where the medium is continuously fed through the capillary containing the biofilm, a medium of 1/10 the strength of the inoculation medium was used.

Flow Cells

Biofilm samples for these experiments were grown inside square, glass, capillary flow cells of one millimeter cross section which were attached to feed and waste carboys to allow for gravity driven nutrient flow. Square capillaries were used so that the work done for this project could dovetail with other research sponsored by the W.M. Keck Foundation using confocal laser microscopy which requires that samples are viewed through a flat glass surface. A one millimeter square capillary was used for this work because the technique for growing biofilms in this system is well documented at the Center for Biofilm Engineering (CBE) and it is used for many of the confocal microscopy experiments.

Biofilm Growth Setup

Structural Images and Fluid Flow Data

The biofilm was grown inside the flow cells described above. After inoculating standard growth medium with a frozen culture, the cells were allowed to grow overnight for 16-20 hours in a shaker incubator at a temperature of 37°C. The overnight planktonic culture was then used to inoculate the flow cell shown in Figure 2.1 for four hours. After allowing the cells to settle out and attach to the surface for the four-hour period, the flow of nutrient was started. The biofilm was then allowed to grow for 48 hours with a nutrient medium flow rate of about 1.7 mL/min which was established using a gravitational head between the feed and waste carboys.

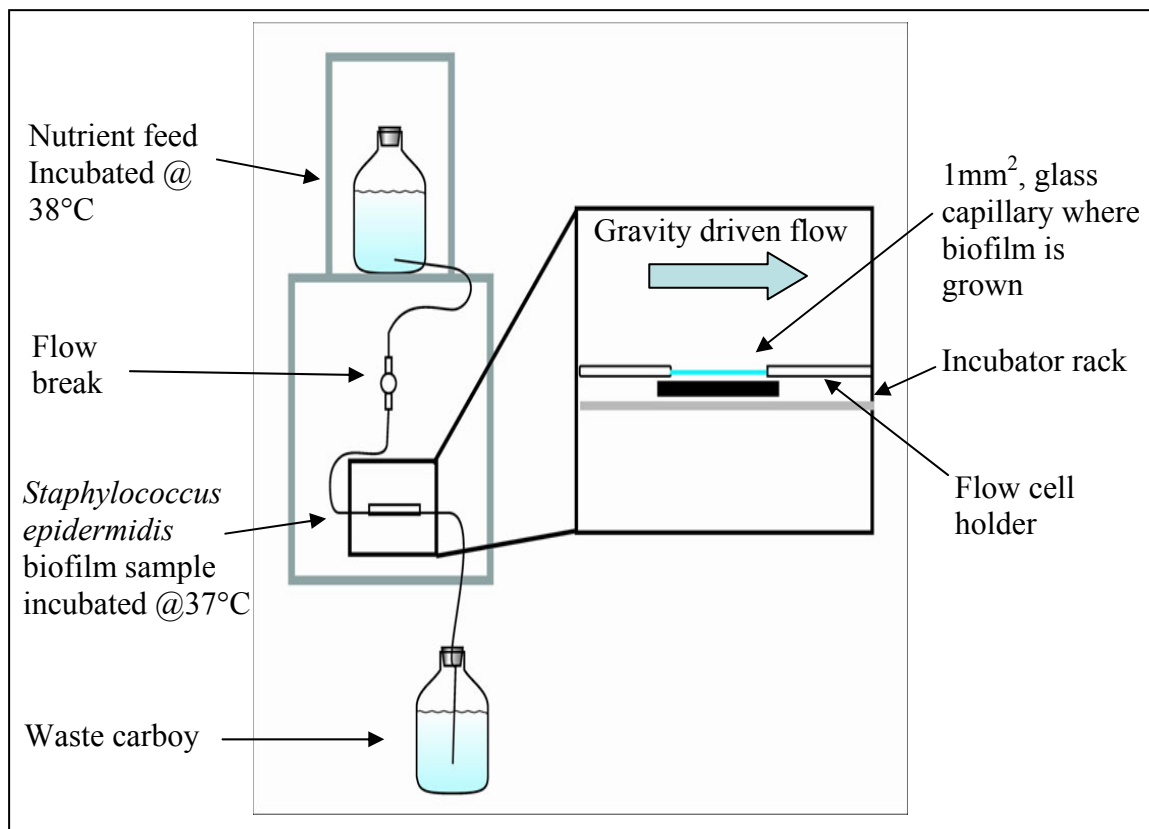


Figure 2.1. Setup for Growing Biofilms in Flow Cells. The *Staphylococcus epidermidis* biofilm sample requires incubation at 37°C for optimum growth. After a four-hour inoculation period the sample is allowed to grow with nutrient flow for 48 hours at a rate of approximately 1.7 mL/min.

Time Lapse Imaging

To acquire images of the biofilm as it was growing the sample had to be grown inside the NMR magnet rather than the CBE laboratory. For these experiments, the initial inoculation was the same with a four-hour period of no flow to allow the cells to attach to the flow cell surface. The inoculated culture was then taken to the NMR lab where it was loaded into the magnet using the setup described below in the NMR Experiment Setup section. The nutrient feed was pre-heated by running the silicone tubing through a water bath at 45°C and was fed through the capillary at a flow rate of

1.7 mL/min. The nutrient feed was heated to 45°C to account for cooling that occurs between the time the fluid in the tubing leaves the water bath and enters the capillary. The sample in the magnet was maintained at 37°C using the temperature control system for the gradient coil. The gradient coil is heated by a water bath which pumps the warm water through the coil and recycles it back to the bath.

NMR Imaging System

A Bruker Avance DRX spectrometer along with a 250 MHz superconducting magnet was used to conduct the NMR imaging experiments. Micro-imaging was accomplished using a Bruker Micro5 probe and gradient amplifiers to produce magnetic gradients up to 2 T/m. An rf coil with a internal diameter of 5 mm was used for all the NMR experiments presented. To acquire images and process diffusion and velocity data the Bruker imaging software package (Paravision) was employed. Additional data analysis to obtain velocity maps was performed using MatLab (MathWorks, Inc.).

Contrast agents were added to the feed at a concentration of 0.6 mL per liter nutrient feed in order to decrease the experiment times. A common contrast agent, copper sulfate, caused the biofilm to slough off so experiments using this contrast agent were abandoned. However, low concentrations of Magnevist have been shown to have no affect on the growth of the biofilms [11] so it became the contrast agent of choice. Magnevist is metabolically inert because the gadolinium metal, which effects the relaxation times in a sample, is chelated with diethylenetriamine pentaactetic acid (DTPA) to form a stable gadopentetate dimeglumine salt.

NMR Experiment Setup

To obtain NMR data for these experiments the capillary containing the biofilm sample was loaded into the NMR magnet as shown in Figure 2.2. The pressure head between the carboys was used to manipulate the flow through the capillary. The various experiments described in the next sections were then conducted to image biofilm structure and the fluid dynamics within the capillary.

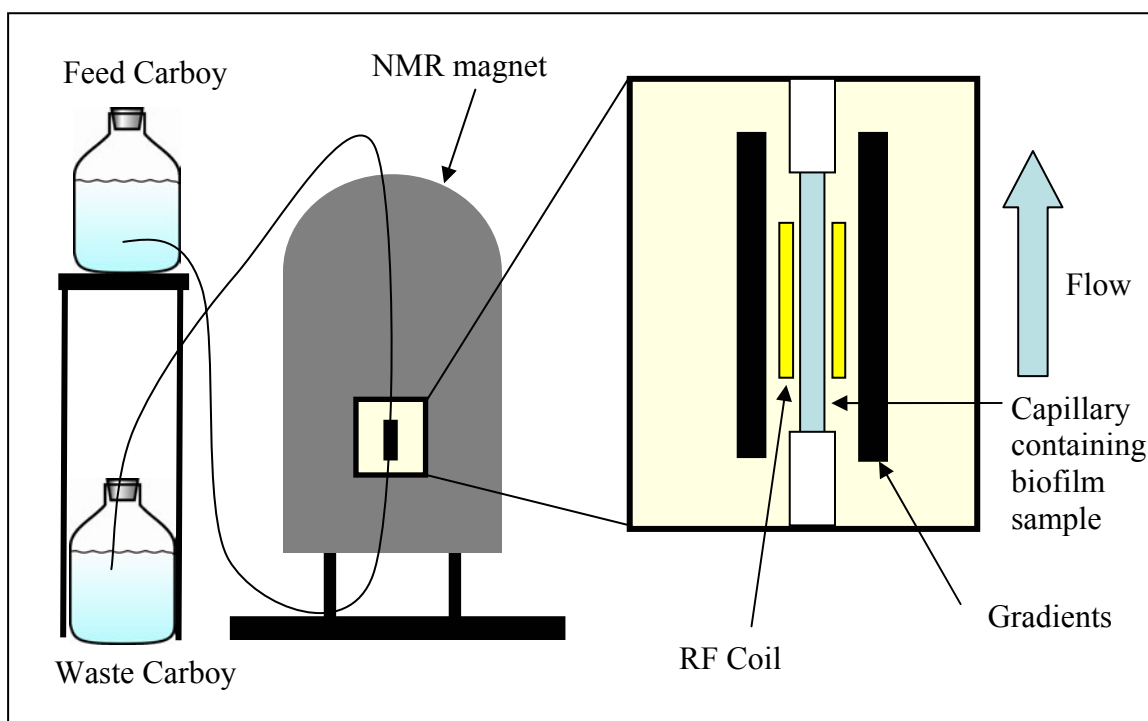


Figure 2.2. Setup for NMR Imaging System. The biofilm sample is loaded inside the imaging probe which is then loaded inside the super conducting magnet. A height difference between the waste and fee carboys controls the flow rate. The direction of flow runs up the magnet, as illustrated, to facilitate the removal of air bubbles from the capillary.

T₁ Weighted Images

T₁ weighted images rely on differing spin-lattice relaxation times for protons in different environments. In general, protons in a more solid-like substance will have a

shorter T_1 and the more fluid-like, the longer the T_1 . This basic principle allows for the imaging or the contrast between protons bound up in a biofilm structure and those in the free, bulk fluid. This technique for imaging the biomass was used in conjunction with the copper sulfate contrast agent. The basic pulse program used in this work to acquire a T_1 weighted image is shown in Figure 2.3. A repetition time (TR) of 200 ms and an echo time (TE) of 10 ms were used with 16 averages. TR is chosen to be short so the biofilm restricted protons will relax to equilibrium and give stronger signal than the longer T_1 for the free water protons.

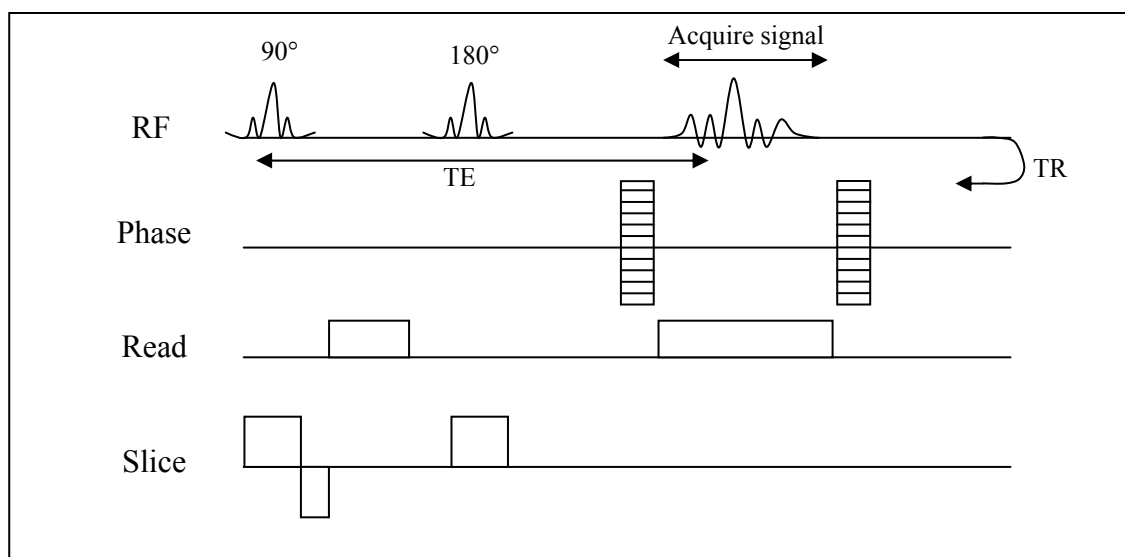


Figure 2.3. Pulse Program for T_1 Weighted Images. The main components of standard NMR imaging sequence are shown. The slice gradient is used to excite spins in the slice of interest while the read and phase encoding gradients provide the spatial resolution. The rf coil is used to both to excite the spins and then detect the signal.

T_2 Maps

T_2 maps are based on the same basic idea as T_1 contrast in that the biofilm bound water will relax quicker than the free water in the bulk fluid. A slice-selection 2-D multi-

spin echo imaging sequence was used with the following parameter values: $TR=500$ ms, $TE=10$ ms, number of echo images=8 (pulse program shown in Figure 2.4). Acquisition time for each map was 8.5 minutes during which time the flow would be stopped. TE can be varied to provide T_2 weighting in the images and a CPMG scheme is used [30].

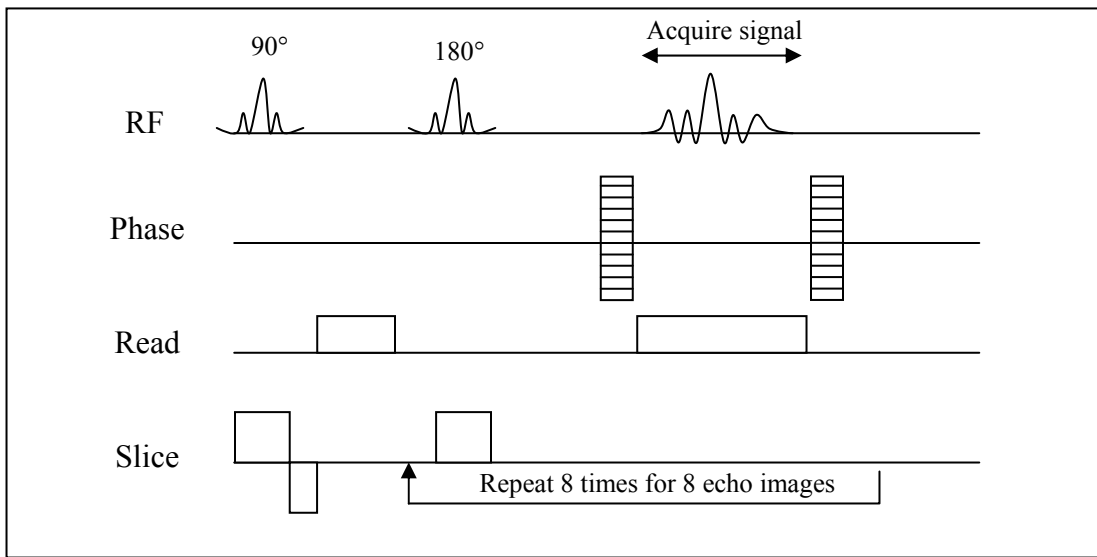


Figure 2.4. T_2 Map Pulse Program. The same basic sequence is used for both T_1 weighted images and T_2 maps. To make T_2 maps the echo image is reproduced 8 times at different values of TE generated using a CPMG method, so that the calculations shown in Figure 2.5 can be performed.

Making a T_2 Map

T_2 maps provide an image based on the contrast of having different T_2 relaxation times for biofilm and free water. In order to construct a T_2 map the experiment described above is performed and a set of images like the black and white panels shown in Figure 2.5 is taken. For the corresponding pixels in each image a graph of TE versus signal intensity is constructed and a T_2 contrast value is calculated as shown in Figure 2.5. The T_2 calculated for each series of pixels is displayed in a single image as show in last panel

of Figure 2.5. All these calculations can be easily performed in the Paravision software in the image display window under the processing tab and using function fitting.

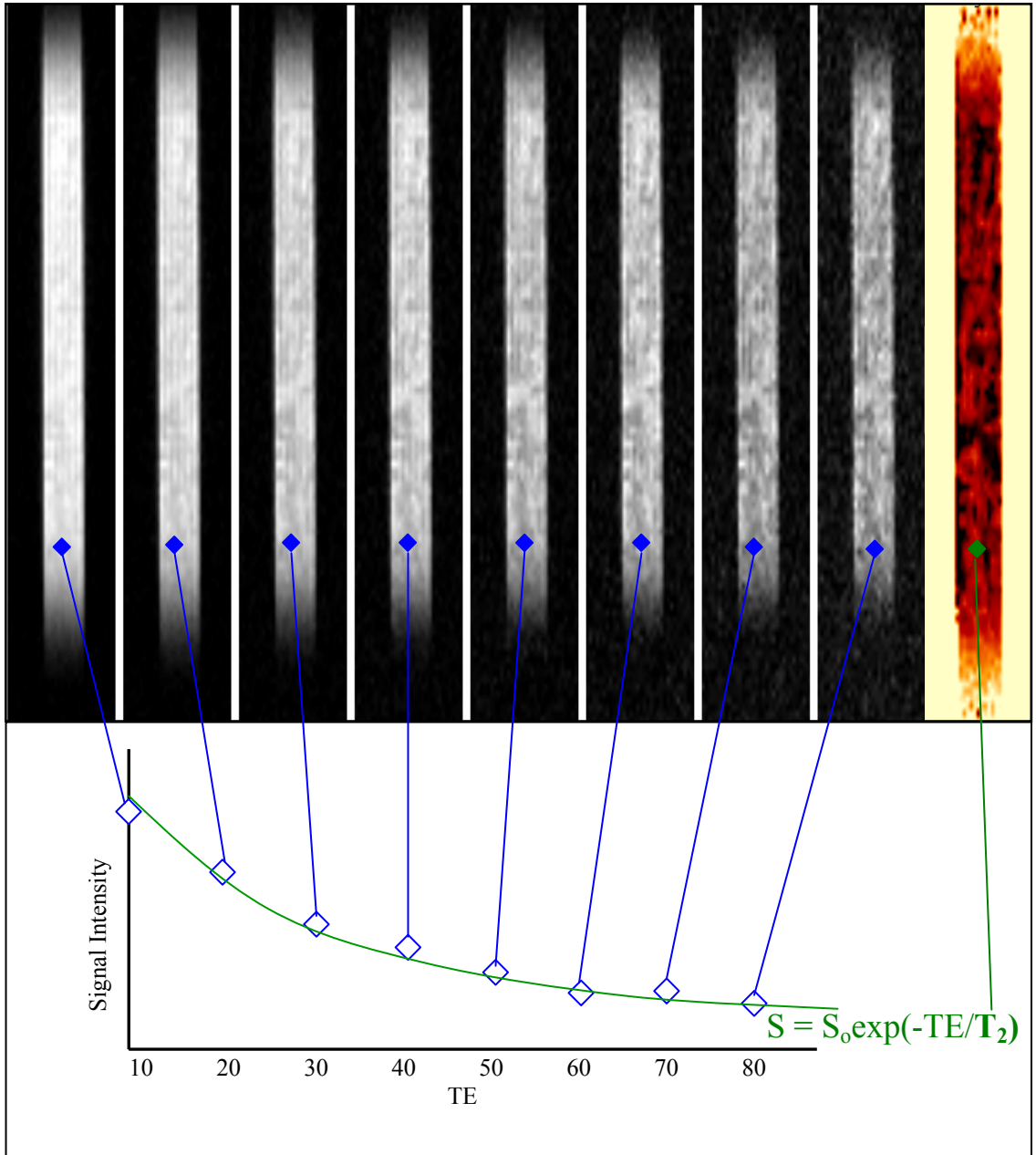


Figure 2.5. Making a T₂ Map. For every pixel in the image the corresponding series of pixels is graphed to determine the T₂ value for that pixel. The T₂ value of every pixel is then presented as a map, shown by the colored image.

Velocity Maps

Velocity data were taken using a Bruker DWI_SE sequence with parameters of: TR = 2000 ms, TE = 20 ms, $g = 0$ and 100 mT/m, $\delta = 1$ ms, $\Delta = 4$ ms, and an acquisition time of 17 minutes. The pulse program in Figure 2.6 shows the bipolar gradient pair which encode for flow detection.

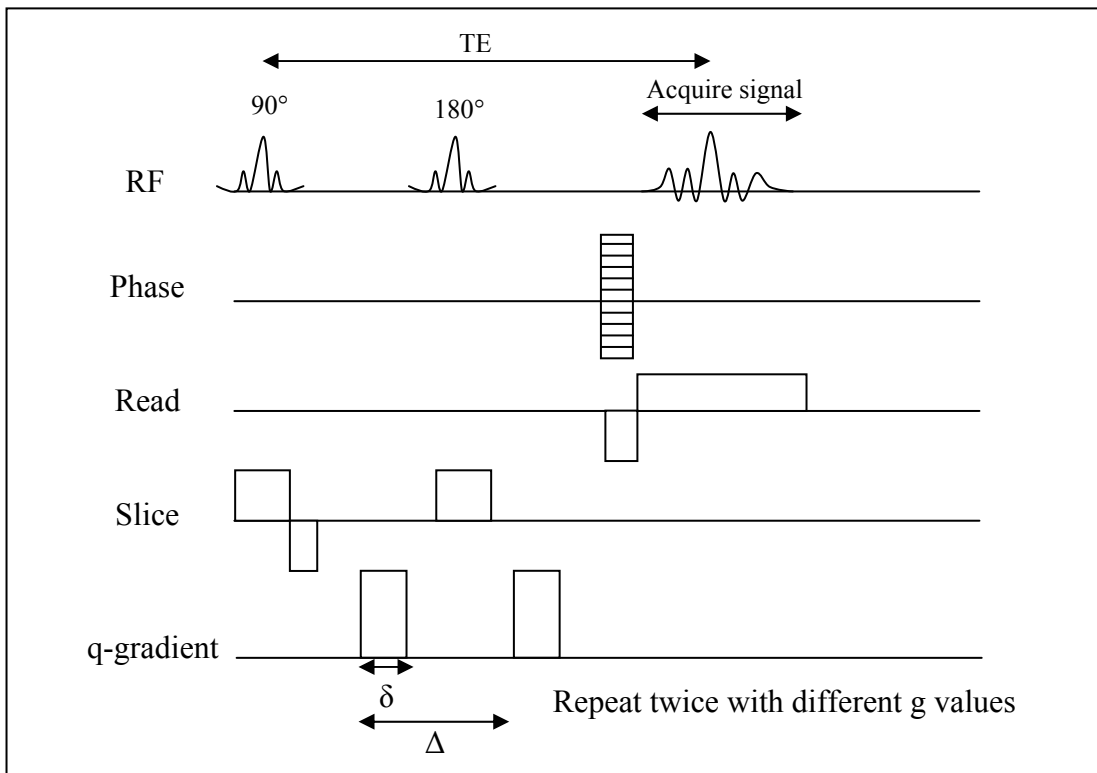


Figure 2.6. Pulse Program for Velocity Encoding. A pair of bipolar gradients is applied on top of the standard imaging sequence in order to detect velocities. The experiment is run twice, once with $g = 0$ mT/m then with $g = 100$ mT/m, and the velocity map is constructed as described in the next section.

Making a Velocity Map

In order to construct a velocity map, the phase images taken at the two different gradient values are needed; these are illustrated in panels A and B of Figure 2.7. One image is then subtracted from the other and then a value of positive and negative π (± 3.14) is added to each pixel value in the image. This produces a map of the velocity as shown in panel C of Figure 2.7.

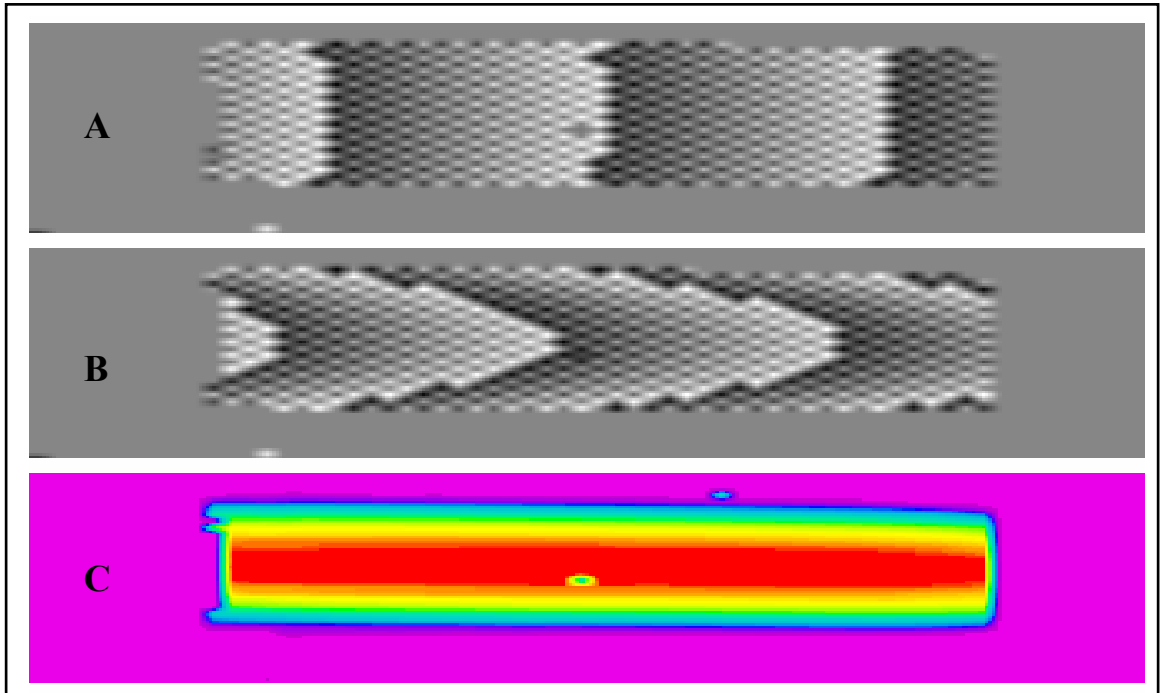


Figure 2.7. Making a Velocity Map. Images of the velocity profile (panel C) are made by subtracting the phase image A from B. The phase image in panel A is from the gradient value of 0 mT/m and image B is from a gradient value of 100 mT/m.

Propagators

Propagator data were acquired using a pulsed gradient spin echo (PGSE) [21] sequence shown in Figure 2.8. The parameters used were $\Delta=15$ ms, $\delta=2$ ms and 128 gradient value steps from -1000 to 1000 mT/m in order to fully sample q -space and avoid truncation artifacts [31].

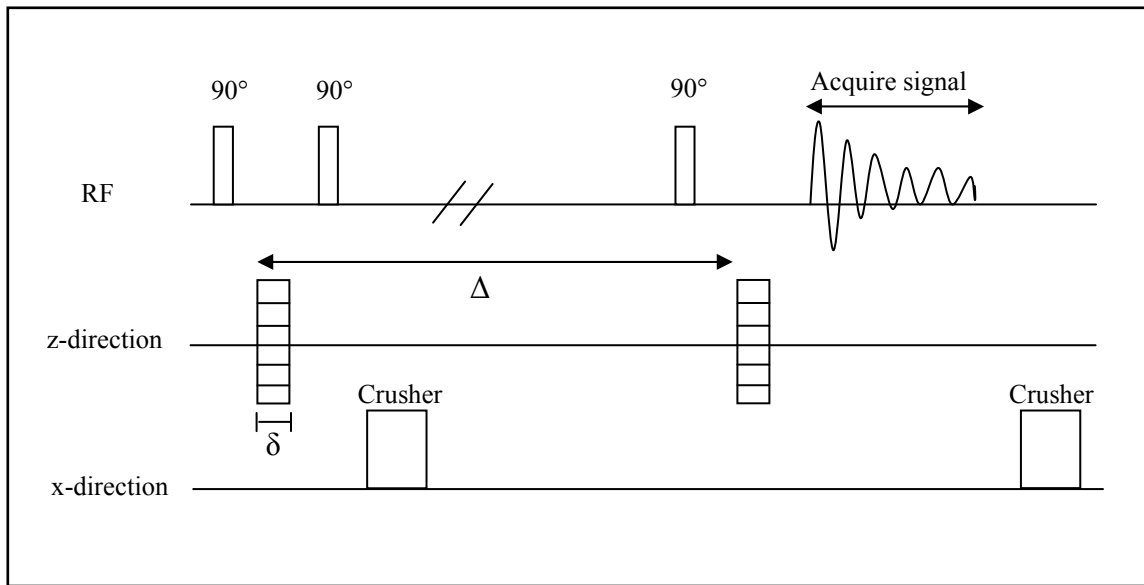


Figure 2.8. Propagator Pulse Program. The above sequence samples all the way out to edges of q-space in order to obtain all the data points needed to construct propagators.

RESULTS AND DISCUSSION

Clean Capillary

Theoretical Solution for Flow in Square Capillary

Laminar flow in a square capillary can be described by the conservation of momentum equation for a Newtonian, incompressible fluid [32]. In order to simplify the equation for the specific case of a square capillary, the system is assumed to be at steady state, resulting in a cancellation of the time dependent derivatives. Also, the flow is assumed to be laminar and the components of velocity in the x- and y- directions (v_x and v_y , respectively) are assumed to be zero and by the equation of continuity the change in the z-component (v_z) of velocity is zero in the z-direction. The resulting equation of the form of Poisson's equation, relates the Laplacian of the z-component of velocity to the axial pressure gradient:

$$\mu \left(\frac{\partial^2 v_z}{\partial x^2} + \frac{\partial^2 v_z}{\partial y^2} \right) = - \frac{dP}{dz} \quad (3.1)$$

where μ is the viscosity, and P is pressure.

Equation 3.1 can easily be solved using the partial differential equation solver in MatLab (PDE Toolbox) [32], to generate the graph shown in Figure 3.1. The graph demonstrates that the theoretical solution predicts that the velocity will be maximal at the center of the capillary and zero at all the sides due to the imposed no-slip boundary condition. This solution also shows that the flow in the corners of the capillary is nearly zero resulting in a solution that is very similar to that for a circular tube. The non-axial

components of velocity were assumed to be negligible for this case, so only the z-component of velocity is graphed through the cross-section of the capillary.

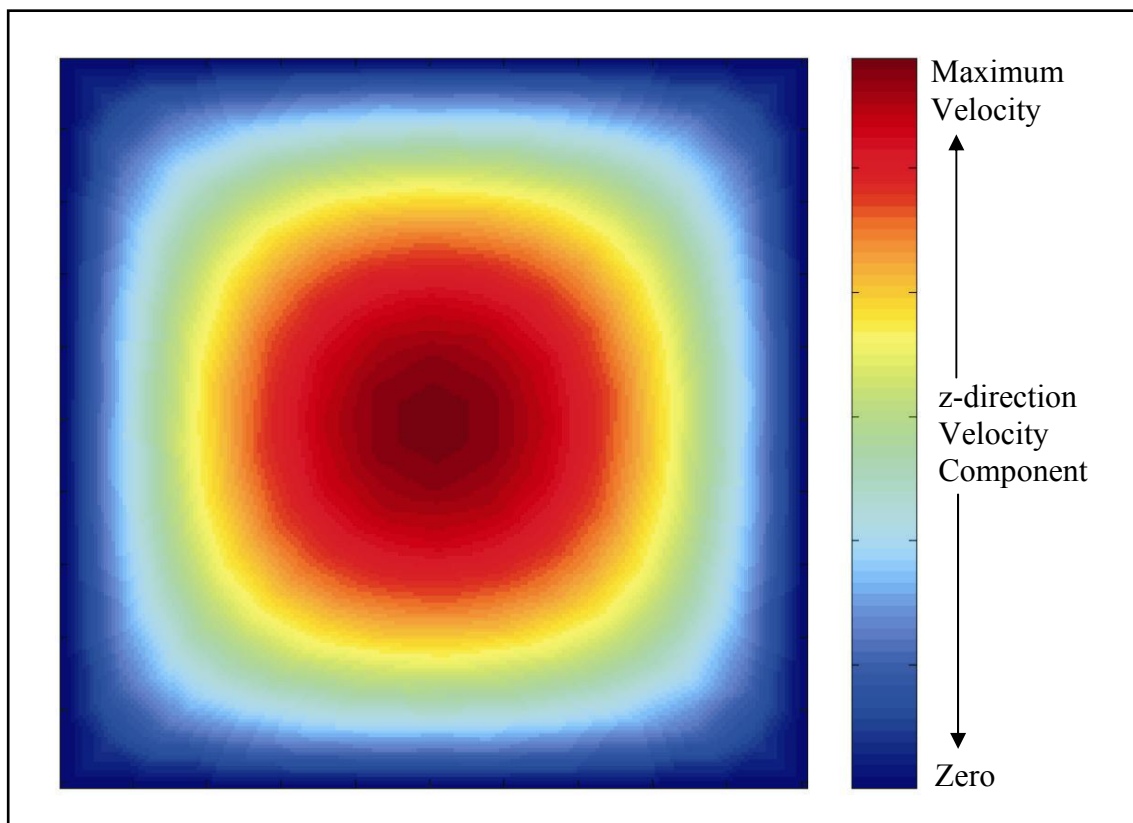


Figure 3.1. MabLab Solution for Flow in a Square Capillary. A graphical representation of the theoretical solution to the conservation of momentum equation for flow in a square duct is shown. The velocity is a maximum at the center and zero at the walls with areas of very slow flow in the corners.

NMR Measurements of Flow in a Clean Capillary

Velocity data in a clean square capillary at a low Reynolds number of $Re = 16$ were collected to compare with theoretical calculations. A visual observation of the theoretical profile in Figure 3.1 and the measured profile in panel B of Figure 3.2 show good agreement. A more quantitative representation of this observation is presented in Figure 3.3 with a graph comparing a transect of velocity data through the center of the

capillary in both cases. The graph illustrates that the NMR velocity data correspond directly with a theoretical profile for laminar flow.

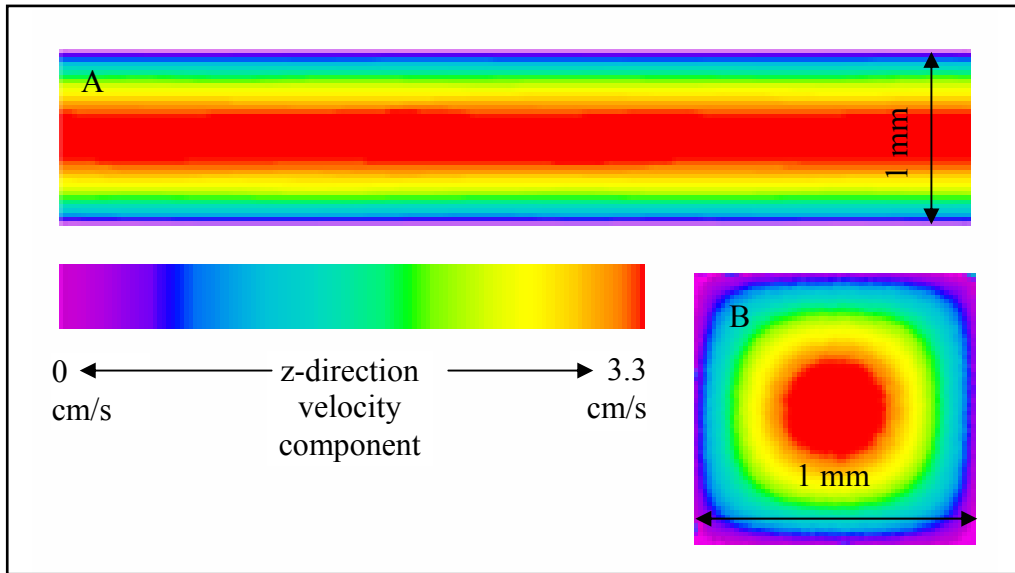


Figure 3.2. NMR Velocity Maps in a Clean Capillary. NMR provides velocity maps in both the longitudinal and transverse directions that are in direct agreement with solutions to the conservation of momentum equation. Figure reprinted from Seymour et al. [5].

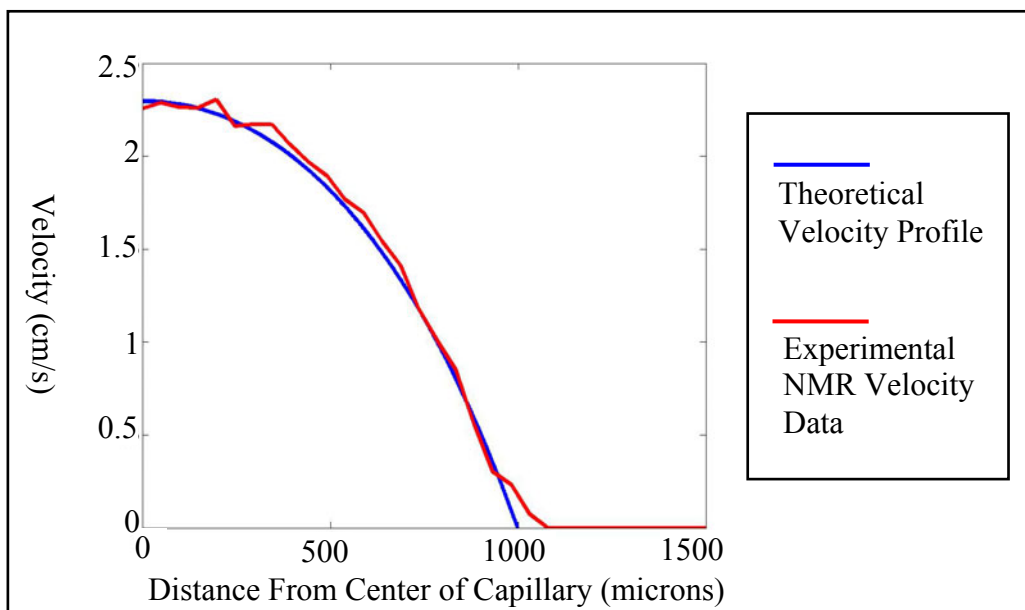


Figure 3.3. Comparison of Measured and Theoretical Velocity Data. This graph demonstrates that the NMR data and theoretical simulations produce very similar results. The profile in both cases is uniform and symmetric.

The NMR measurements for Figures 3.2 and 3.3 were collected with the velocity encoding sequence presented in the Materials and Methods section. The NMR measurements provided not only a visualization of the velocity profile in the transverse direction but also in the longitudinal cross section shown in panel A of Figure 3.2. Attempts to measure x- and y- components of velocity proved that they were not present in the clean capillary. This result is congruent with the assumptions that the z-component of velocity is the only significant one in a laminar flow system which is made when solving the conservation of momentum equation.

Biofilm Fouled Capillaries

Structural Images of Biofilms

Copper Sulfate Contrast Agent. The first images of biofilms acquired at the MSU NMR Imaging laboratory used copper sulfate solution as a contrast agent. A T_1 weighted NMR imaging technique was employed to produce the images shown in Figure 3.4. The darker areas in the images are areas with a shorter T_1 relaxation time indicating that the water molecules are restricted and biofilm is present. These images demonstrate one of the advantages of the NMR technique in that the capillary can be sectioned at any point within the capillary. This allows images to be taken throughout the entire depth of thick biofilms which is not possible with any kind of light or laser microscopy technique.

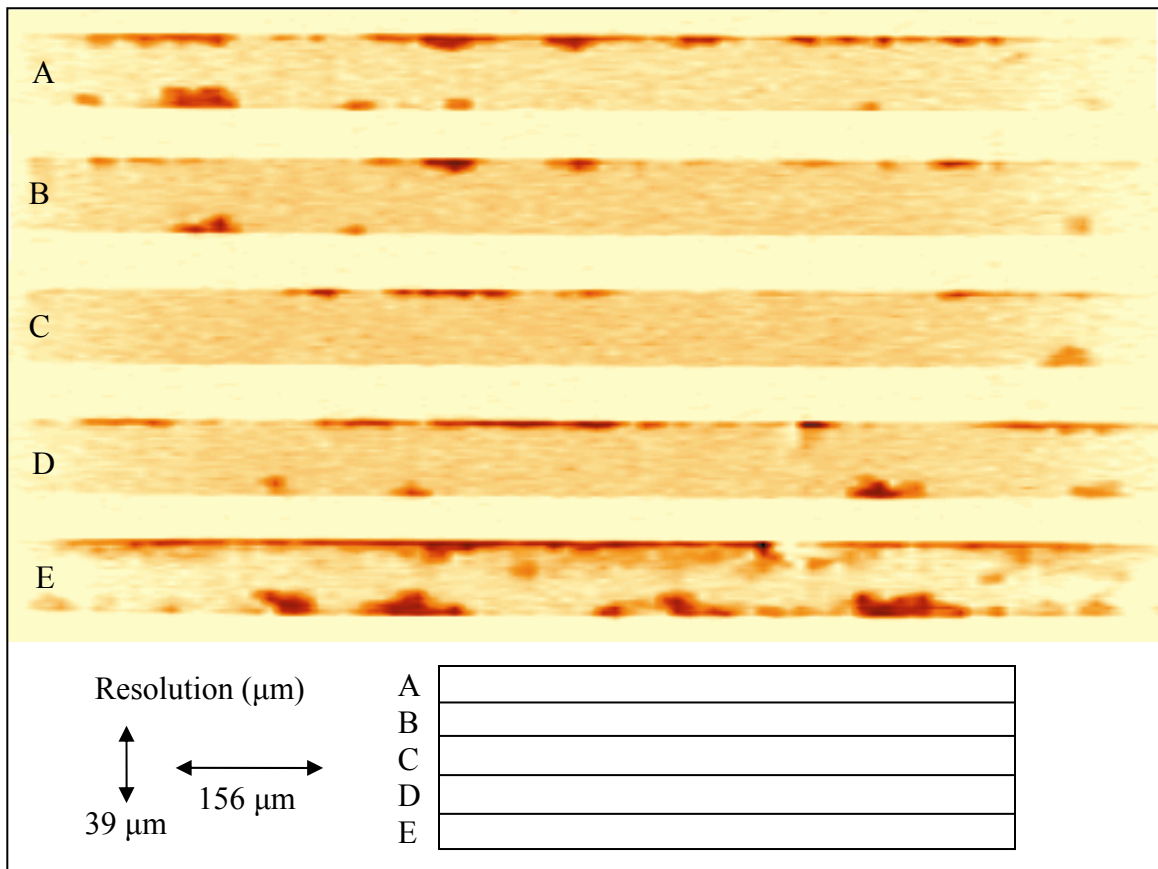


Figure 3.4. NMR Images with Copper Sulfate Contrast Agent. Copper sulfate solution was used as a contrast agent to acquire this set of T_1 weighted images. Each image is of a slice $200\ \mu\text{m}$ thick and the dark areas indicate biomass.

This method of visualizing the biofilm did produce images; however, there were some problems with using copper sulfate. The main drawback was that the biomass would stain blue after a day of exposure and the biofilm would begin to slough off. The reason for this problem is not clear but copper is known to be toxic to microorganisms. Another conjecture is that the copper ions disrupted the polysaccharide EPS matrix. Either way, the experiments using copper sulfate were abandoned in favor of a chelated gadolinium solution.

Magnevist Contrast Agent. Since there were problems encountered using copper sulfate, the medical contrast agent Magnevist (Berlex Laboratories), a chelated gadolinium solution, was employed. Magnevist, or gadopentetate dimeglumine, serves the same function as copper sulfate by providing faster experimental times but the Gd^{+++} metal chelate is in the form of a stable salt and is metabolically inert. With this contrast agent a different method of acquiring images was found to provide the best contrast. Maps of the T_2 relaxation time rather than differing T_1 values were used to distinguish between biomass and bulk fluid. Figure 3.5 is a set of images obtained using this method.

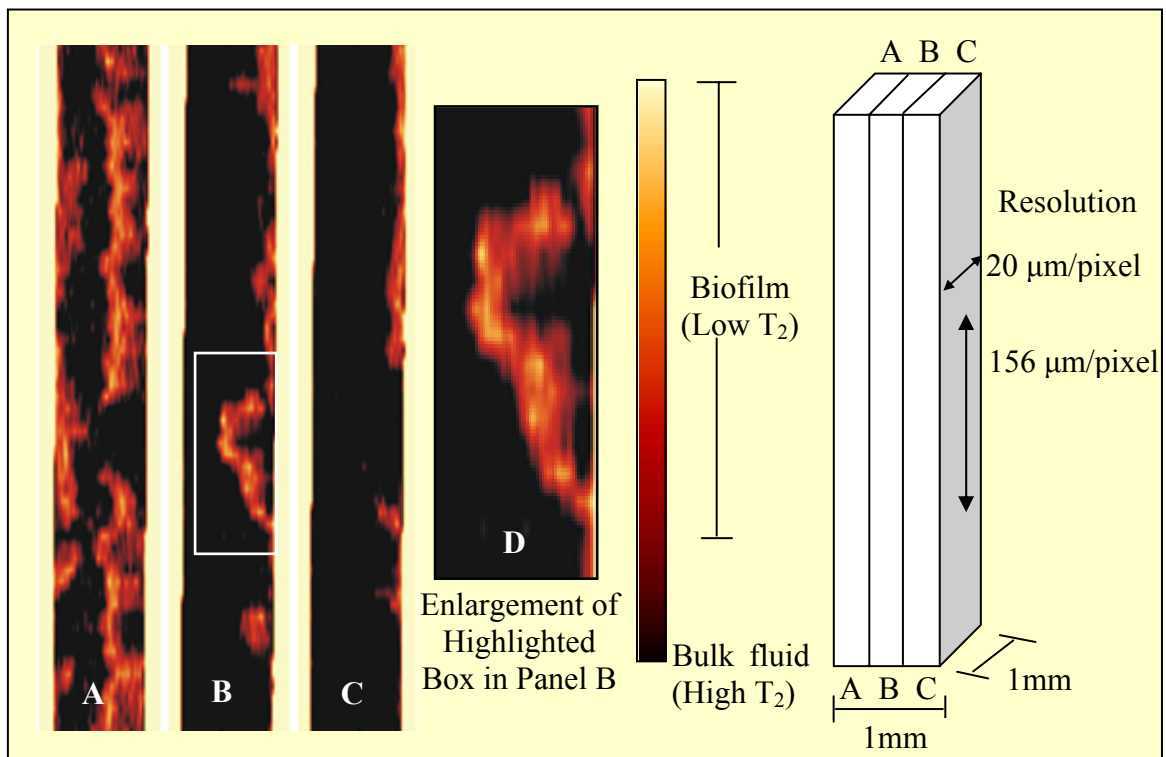


Figure 3.5. NMR Images of Biofilm Structure. These T_2 maps display the complex, heterogeneous structure of a biofilm. There is more biomass present in the image in panel A because that is the wall that was on the bottom when the cells were inoculated into the capillary and allowed to settle. The box highlighted in panel B, which is blown up in panel D, reveals a 'hollow' cluster of biomass like those seen using confocal microscopy. Portions of figure reprinted from Seymour et al. [5].

These T_2 maps display the overall structure of a biofilm, showing a heterogeneous distribution of T_2 relaxation times which is indicative of the complex structure. Areas at the edges of the biomass may be displaying different T_2 values because of partial voluming effects where the pixel is averaged over both biomass and free water. The hollow cluster seen in panels B and D of Figure 3.5 is thought to be caused by cell death in the center of the large clusters as a result of nutrient deprivation. This phenomenon has also been observed using confocal microscopy and with other biofilm species and is predicted by the BacLab biofilm modeling program. These images also display that *S. epidermidis* biofilms are attached only in areas where the bacteria were able to settle out since the bacteria are a non-motile species.

With Magnevist as a contrast agent, T_2 was found to produce images with the best contrast. Diffusion maps were also collected but the diffusion coefficients within a biofilm were so similar to that of the free water, in agreement with non-spatially resolved data [9], that the contrast between the two was very small. Since diffusion maps provided no advantage over the T_2 maps and diffusion maps take hours to collect and T_2 maps based on a multiple echo CPMG method [30] take only 20 minutes, T_2 maps were used as the imaging technique of choice.

In order to obtain these images the biofilm did not need to be altered in any way and as long as nutrients were available and the temperature kept at 37°C, the biomass would continue growing. However, once the biomass is deprived of nutrients large clumps of cells would begin sloughing off as they died. A set of images showing the result of this sloughing process is shown in Figure 3.6. Images of the biofilm growing

while NMR experiments are conducted are shown in Time Lapsed Imaging section below.

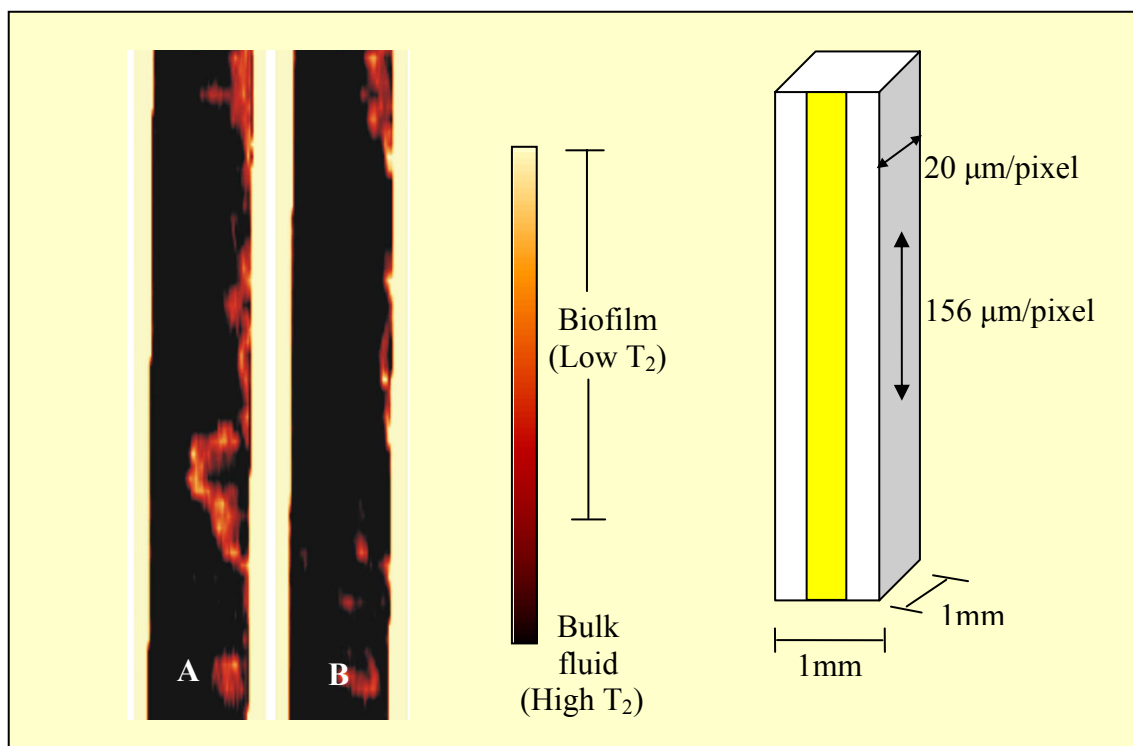


Figure 3.6. Biofilm Sloughing. The biofilm shown in panel A was grown for 48 hours with 1/10 strength TSB in a 37°C incubator. After being deprived of nutrients at a temperature of 25°C for approximately 18 hours the large clump of biomass sloughed off as shown in panel B.

Velocity Maps Around Biofilms

The main focus of this research project was to utilize one of the most powerful aspects of NMR by mapping fluid flow patterns in biofilm fouled capillaries. There is much speculation regarding the fluid dynamics around and within biofilms. NMR allows for the non-invasive detection of velocities and diffusion to investigate the transport and flow patterns in a capillary fouled with biomass. Figure 3.7 displays a series a velocity images in a biofilm fouled capillary that was acquired using NMR.

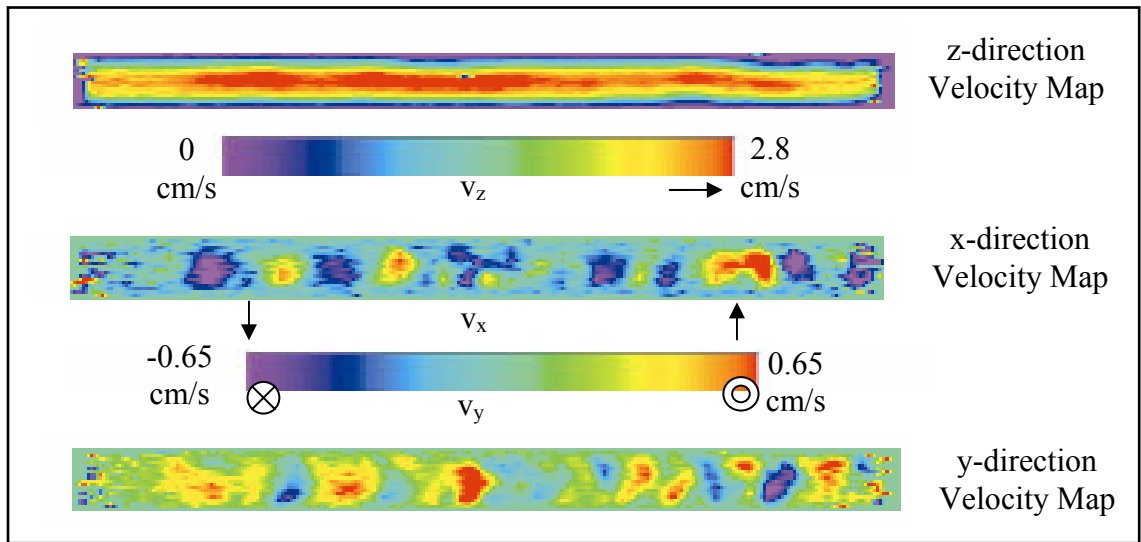


Figure 3.7. Velocity Maps of a Biofilm Fouled Capillary. These maps display the perturbations that occur in the z-direction velocity component as a result of biofilm fouling. In addition, secondary components of velocity, v_x and v_y , are induced which display a distinct periodicity. For v_z , the color red indicates a vector direction to the right, as shown by the arrow, with a magnitude of 2.8 cm/s. For v_x , red indicates a vector direction pointing up with a magnitude of 0.65 cm/s and purple indicates a vector pointing down, or in the negative x-direction, with a magnitude of 0.65 cm/s and is referred to as -0.65 cm/s. The color bar for v_y is similar to that for v_x with red indicating a vector pointing out of the page and purple a vector pointing in the negative y-direction or into the page. These images are through the center slice of a capillary and the T_2 maps did not display any biofilm formation in this slice. These are Paravision images of the velocity maps shown in Figure 3.11.

The velocity images in Figure 3.7 are in sharp contrast to those for a clean capillary (Figure 3.2). The z-component of velocity is no longer uniform and symmetric and secondary (x and y) components of velocity are present. These secondary components are of particular interest since they greatly enhance diffusion and transport within a biofilm by changing the fluid mixing properties from purely diffusive to enhanced convective mixing. An interesting aspect of these secondary components is that they appear to be periodic on a scale of the capillary diameter. In addition, they also appear to be coupled: the x-component is orientated in the negative x-direction when the y-component is orientated in the positive y-direction and vice versa. The major

implication of these non-axial components of velocity is that the conservation of momentum equation is now dependent on an additional two variables making it much more difficult to solve as the problem is now non-linear [27].

Figure 3.9 displays two different slices of velocity images and their corresponding T_2 maps that were generated using Paravision. Orientation for these slices and for Figures 3.10-3.13 is presented in Figure 3.8. In Figures 3.10-3.13, graphs of position versus normalized T_2 signal intensity and velocities are presented as a confirmation of the observation that velocities are zero where biofilm is present. The graphs also display the periodicity of the x and y components and the coupling between them in that one is positive while the other is negative. The T_2 values are normalized against the maximum signal intensity in that slice and the T_2 for water is set equal to zero. For the velocity data in the graphs all three components of velocity are normalized against the maximum in the z-direction so that the x- and y-components are displayed as a percentage of the axial velocity.

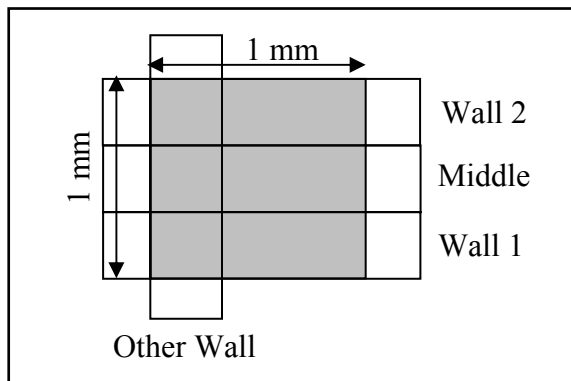


Figure 3.8. Slice Orientation for Figures 3.9-3.13. The data presented in this series of figures are all for the same capillary with the slice selections shown above. Each slice is 0.3 mm thick and is taken in the longitudinal plane, along the length of the capillary.

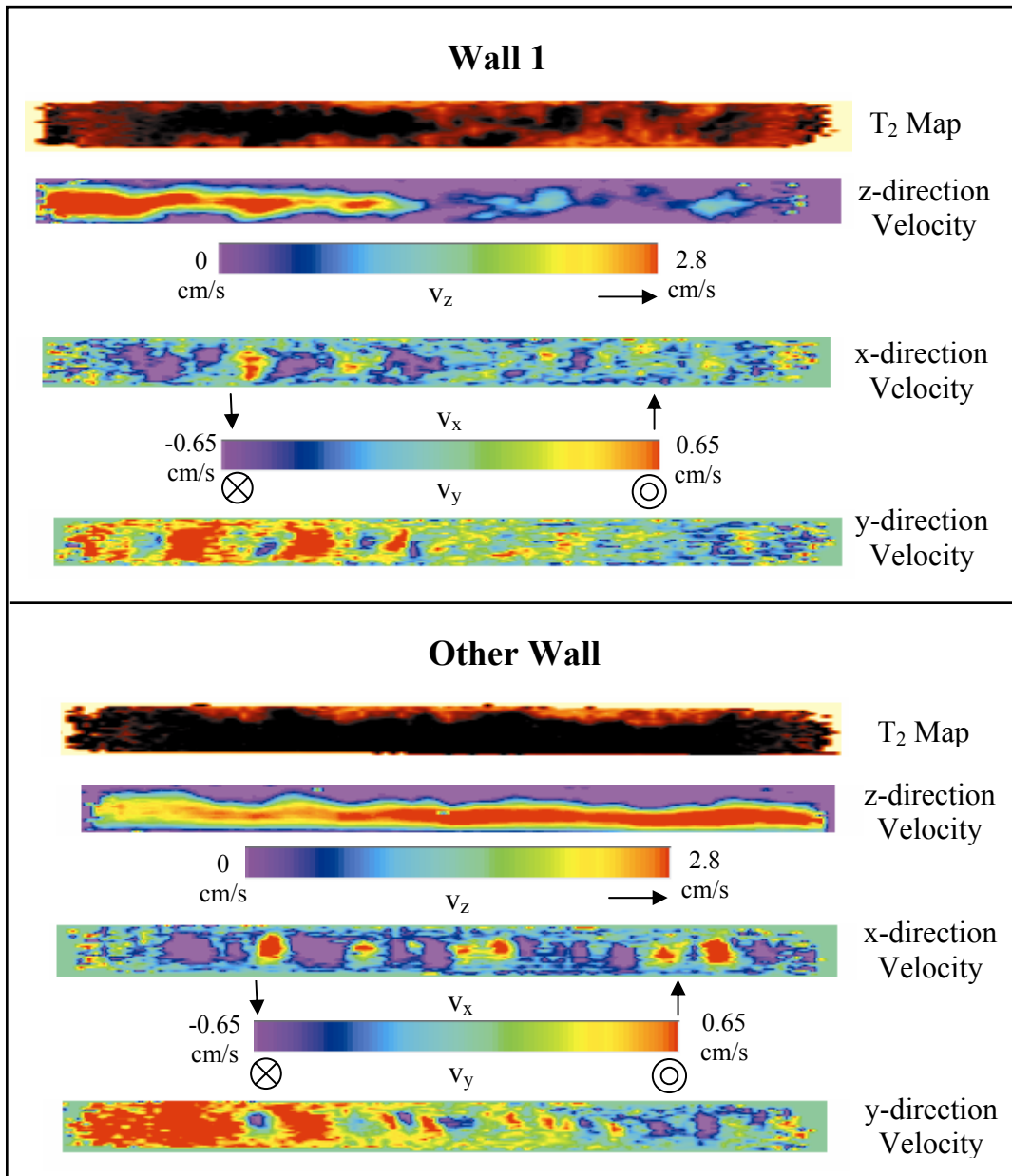


Figure 3.9. Paravision Images of Wall 1 and Other Wall. The T₂ maps from both of these images display areas of thick biomass shown in orange. The color bar for v_z shows that red indicates a vector direction to the right with a magnitude of 2.8 cm/s. For the v_x component of velocity red represents a vector with magnitude 0.65 cm/s that is pointing up. The negative x-direction, shown by the arrow pointing down, will display as purple for a vector with a magnitude of 0.65 cm/s. The v_y component of velocity has the same magnitudes as v_x but red indicates a vector direction coming out of the page and purple a vector pointing into the page. The velocity profiles indicate that there is no bulk flow (v_z) occurring within the biofilm fouled areas. Secondary components (phase and slice velocities) of velocity seem to lose their periodicity and are reduced to random scatter where the biofilm is present.

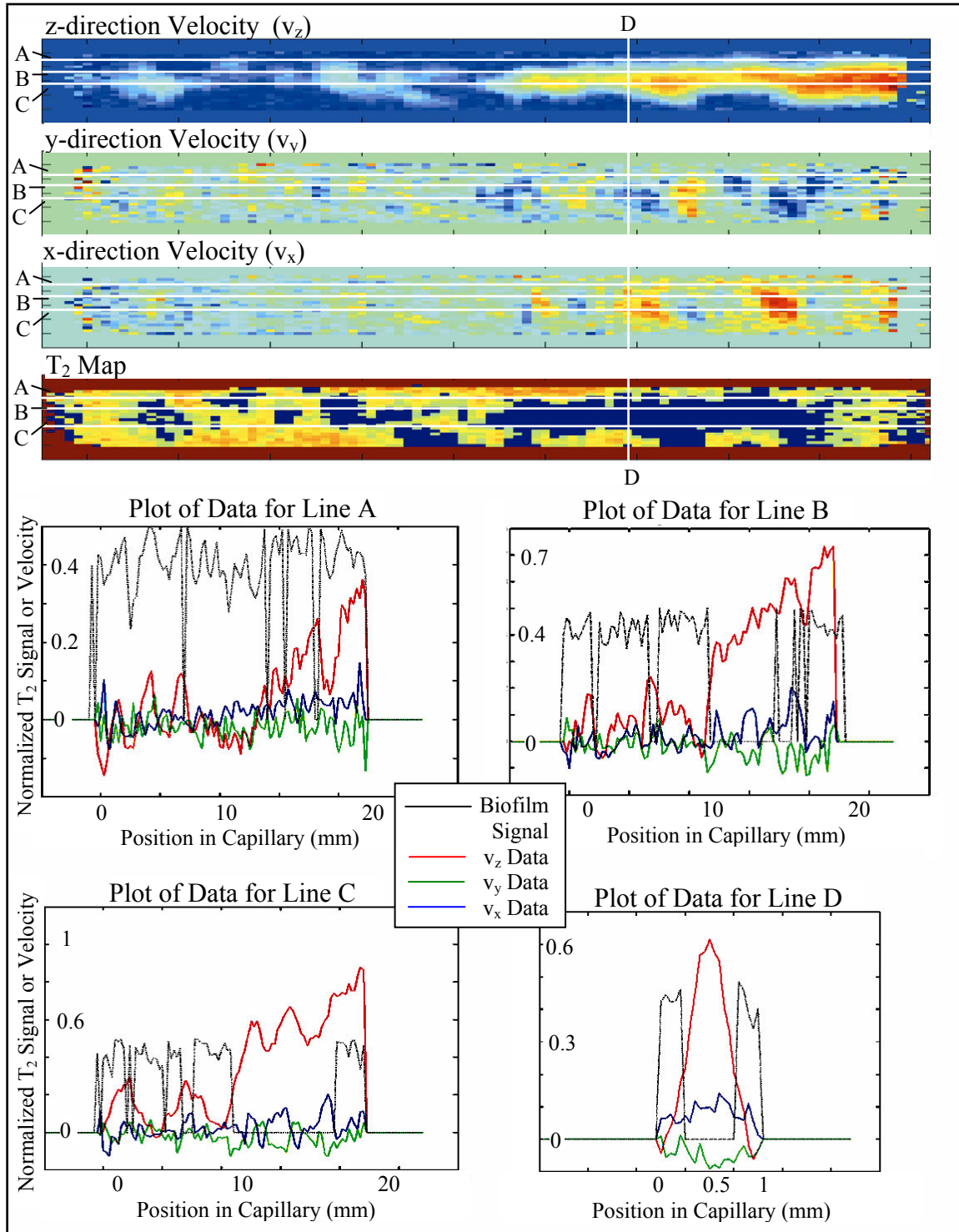


Figure 3.10. MatLab Plots of Slice Wall 1. The lines through the figures shown in the top half of the figure indicate where the lines of data graphed in plots A, B, C, and D. The velocity and T_2 were processed in MatLab for this figure and are shown in order to get a reference of where the lines of data are in the capillary. Refer to Figure 3.9 for a detailed description of the velocity and T_2 maps.

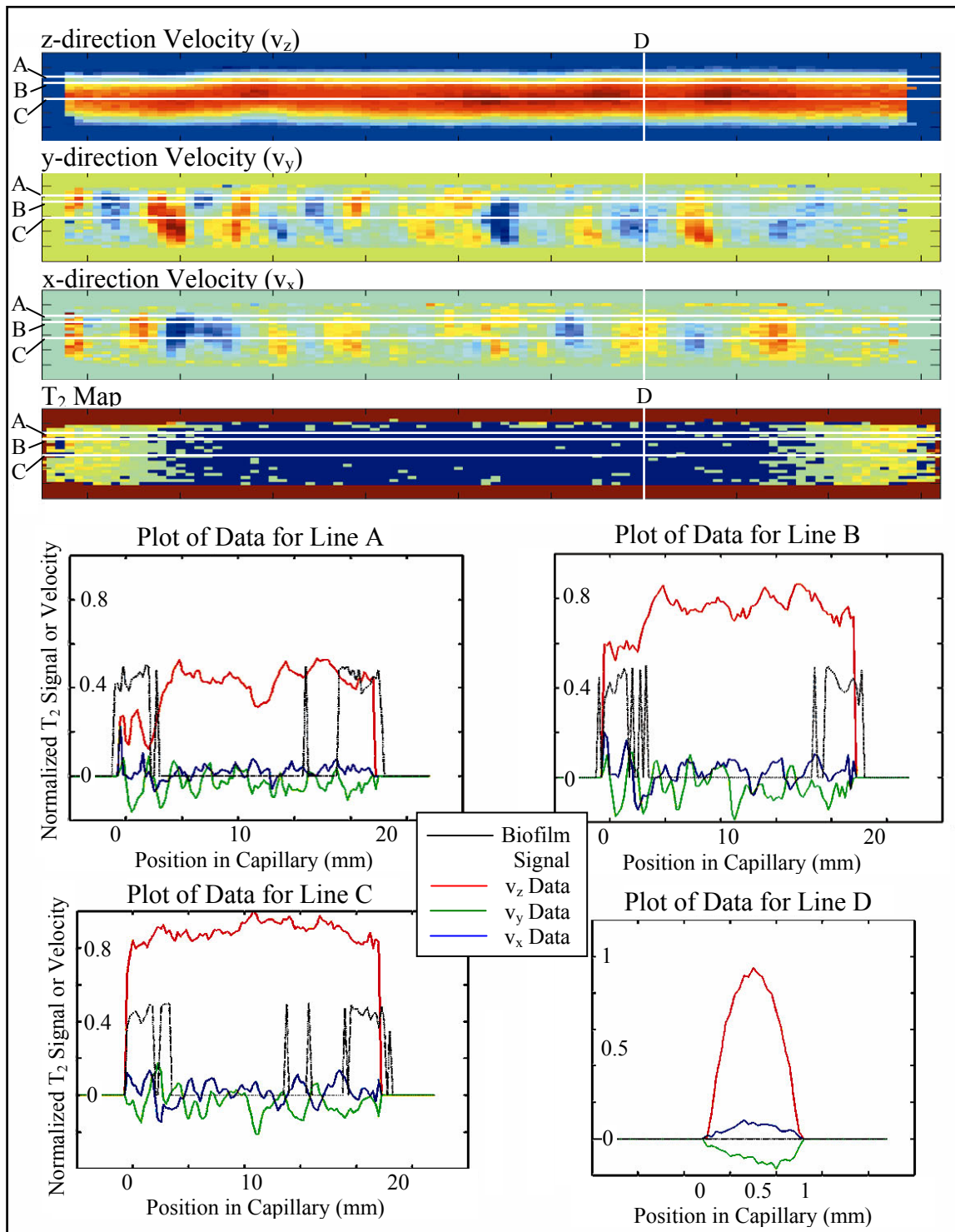


Figure 3.11. MatLab Plots Through the Middle of the Capillary. The plots for this figure demonstrate that in the slice through the middle of the capillary there is very little biomass but the flow is still perturbed from the biomass on the walls. The periodic and coupled nature of the secondary flows is evident from these plots. For a description of the velocity profiles refer to Figure 3.7.

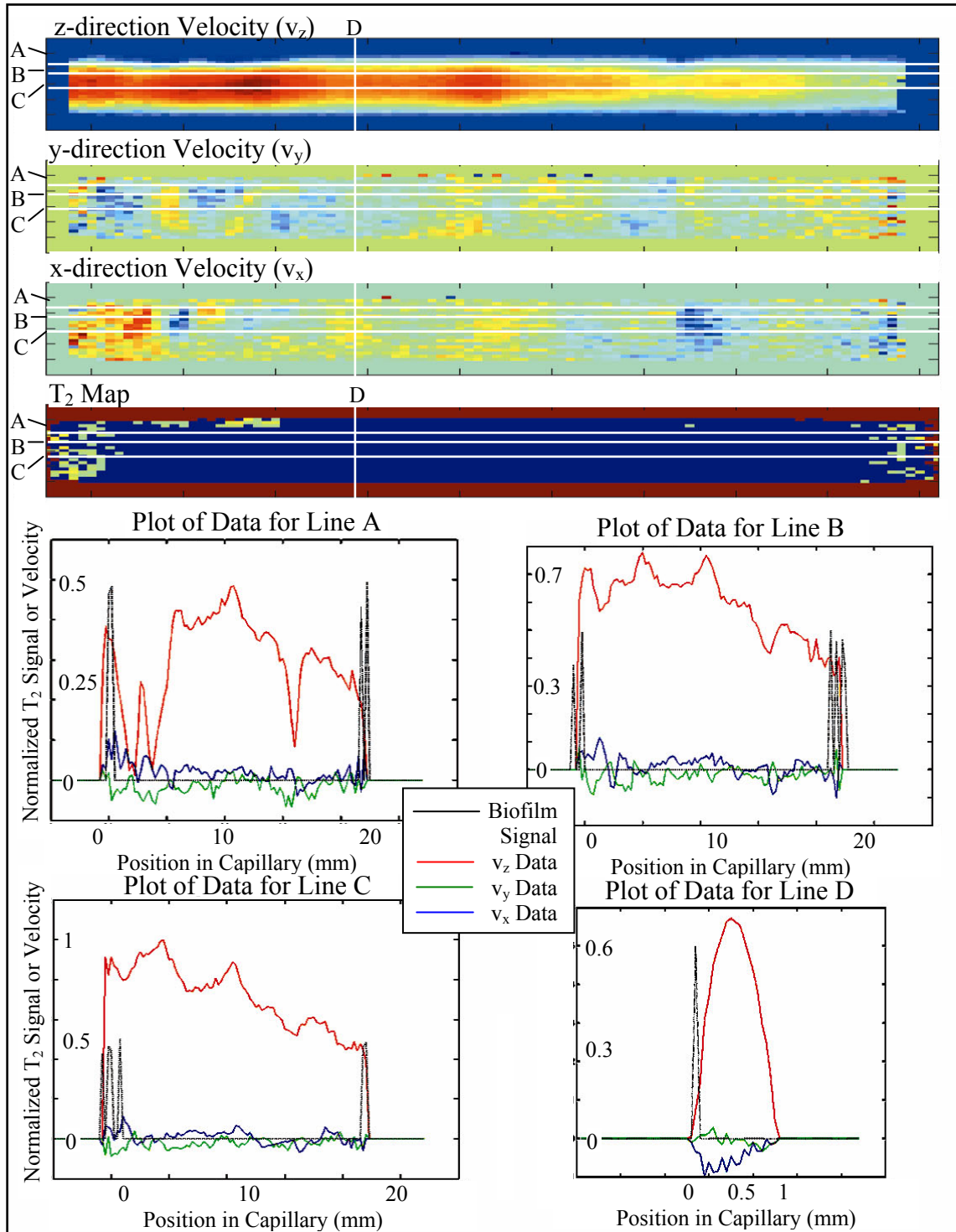


Figure 3.12. MatLab Plots of the Slice at Wall 2. This slice is against a wall which contains almost no biomass and is far away from the majority of the biomass. The plots show that the secondary flows are losing much of their periodicity and coupling in this slice due to the absence of biomass in the neighboring slices. An explanation of velocity profiles is given in Figures 3.7 and 3.10.

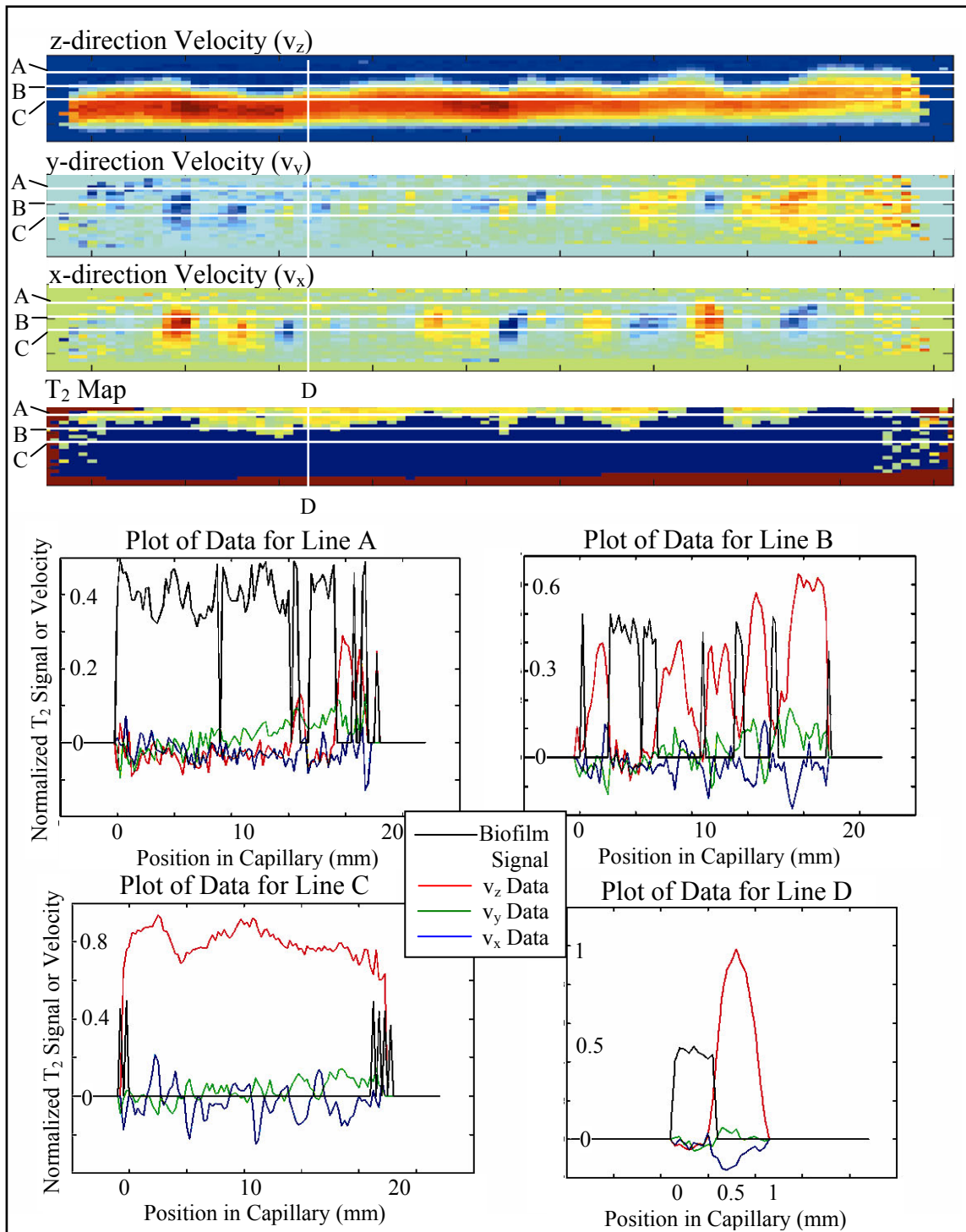


Figure 3.13. MatLab Plots of Slice Against Other Wall. Plots A, B and D demonstrate that in areas where there is biomass present the flows are either zero or negative. Plot C, which is just outside the area where there is biofilm present, is very similar to plots through the middle of the capillary (Figure 3.11). See Figure 3.10 for a detailed description of these T₂ and velocity maps.

Something that should be noted about these Figures 3.10-3.13 is that the areas at the edges of the figures, 0 mm and 20 mm in the longitudinal plots, the data is noisy because it is at the ends of the detection coil and, as such, is not accurate. Nonetheless, there is a multitude of data in the graphs shown in Figures 3.10-3.13 that is accurate and displays some trends which are apparent for every slice. One important trend is that areas that are thick with biomass do not have any positive components of velocity. This trend is presented in the graph shown in Figure 3.14 for the slice against Wall 1. In the areas where bulk fluid is present there is the full range of velocities, from the maximum to zero, while areas of biofilm fouling display primarily zero and small negative velocities. Figure 3.14 demonstrates that the correlations in the quantitative profiles in Figures 3.10-3.13 are representative of the images as it plots all the pixels in the image.

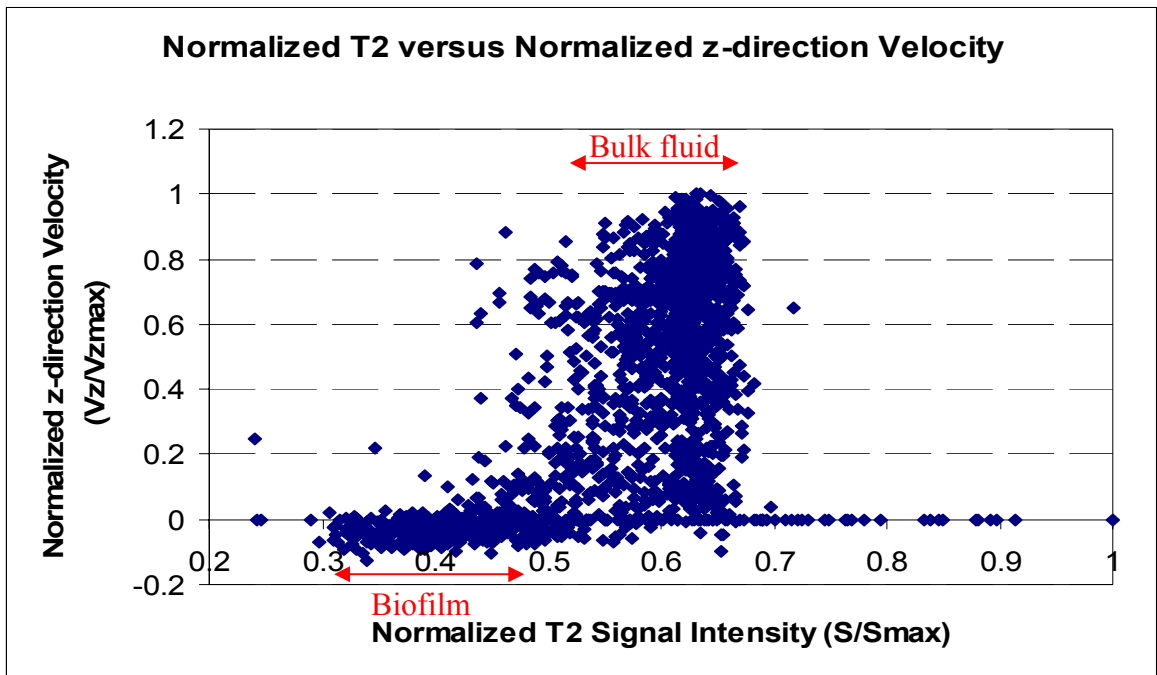


Figure 3.14. T_2 Versus z-direction Velocity for Wall 1. This graph displays that in areas where biomass is present the z-component is primarily zero or negative.

Secondary velocities are significantly impacted by the biofilm being much more variable than the bulk fluid which can be seen in the graphs of Figures 3.10 and 3.13.

Outside of the biofilm, secondary components of velocity, at their maximum values, are about 30% of the magnitude of the z-component. This magnitude is significant and cannot be assumed negligible when deriving equations to explain the flow patterns.

Another aspect of the flow which is most apparent in the graphs presented is the distinct periodicity of the secondary flows. In lines of data that are near the center of the capillary, specifically in plots C and D of Figure 3.11, when one of the components is positive, the other is negative with a crossing of the two at zero. A graph of the v_y versus v_x is shown in Figure 3.15. Since the majority of the points are in the fourth quadrant, the vectors in this slice are orientated in one preferred direction. This indicates that the overall flow pattern within the capillary appears to be helical.

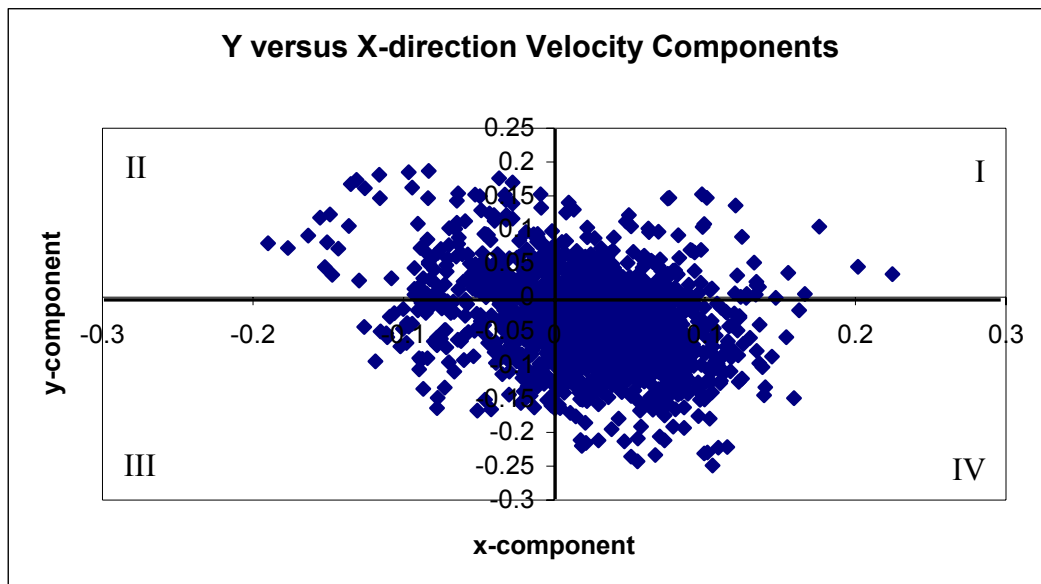


Figure 3.15. Y versus X-direction Velocity Components. This graph shows that the phase and slice components are coupled in such a way as to produce velocity vectors that are preferentially orientated in only one orientation indicating a helical flow pattern.

Time Lapsed Imaging

One of the major advantages of NMR over other microscopy techniques is the ability to non-invasively image living systems without harming the system. This makes NMR perfect for imaging biofilms as they grow. Figure 3.16 displays a set of images acquired over the course of biofilm growth against one of the walls of the capillary. The same capillary sample was used to create the velocity maps in Figure 3.17 but the slice from the center of the capillary was imaged instead of against the wall. These images show the development of the secondary velocity components from 25 to 33 hours after inoculation of bacteria into the capillary.

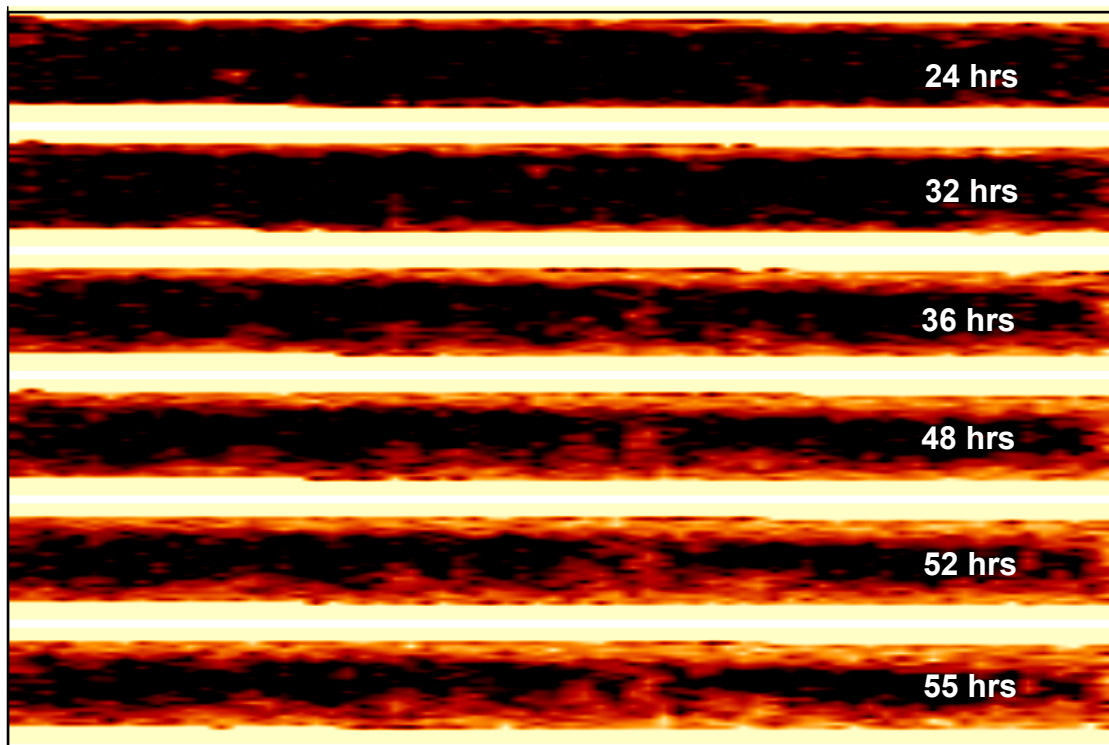


Figure 3.16. Time Lapsed Structural Images. These T_2 maps were collected at different stages in the growth of a biofilm at the time shown after initial inoculation. Orange tones indicate water restricted within a biofilm and black is the bulk unrestricted water.

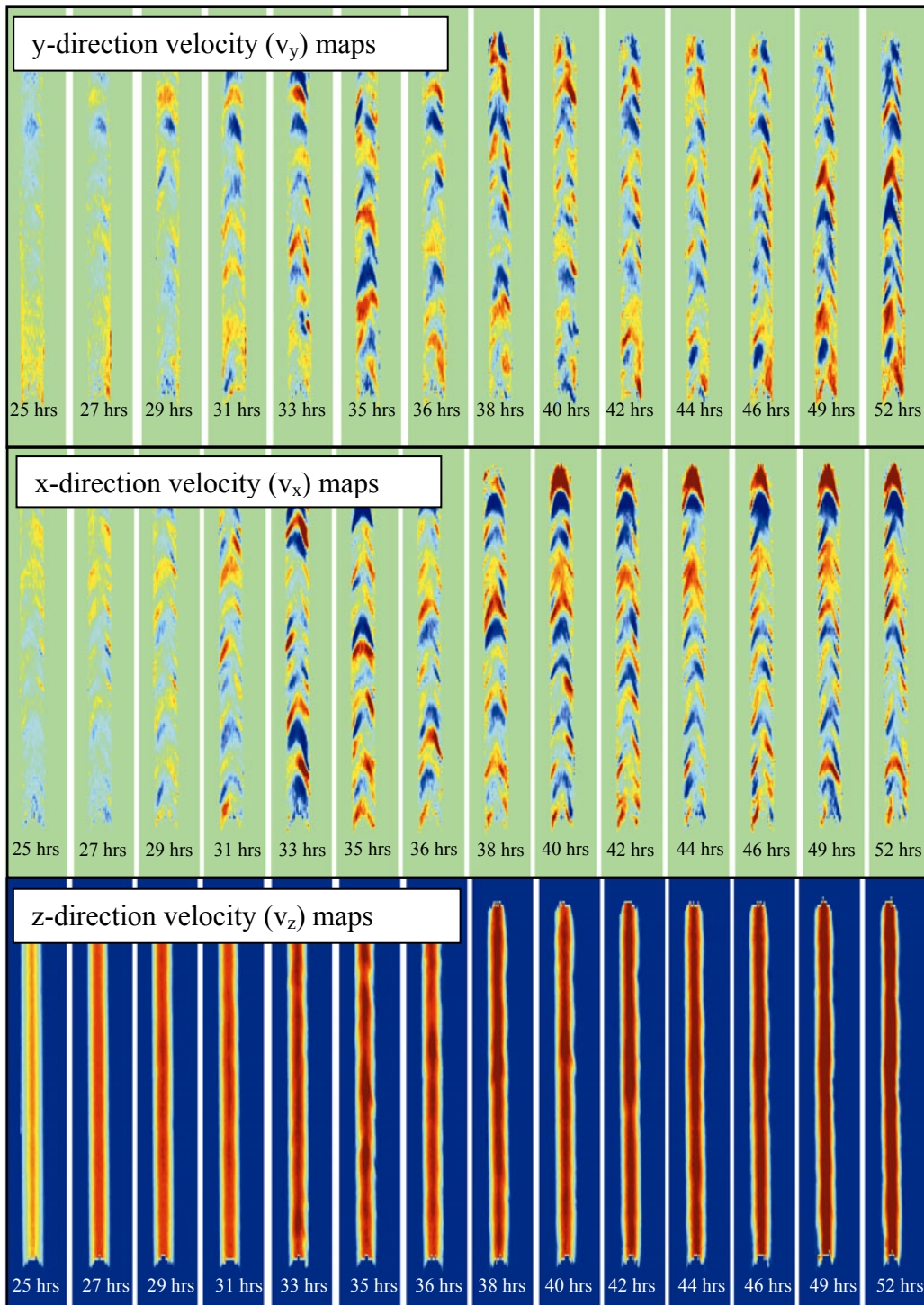


Figure 3.17. Time Lapsed Velocity Maps During Biofilm Growth. The above panels show the development over the course of time of each of the three components of velocity while a biofilm is growing inside the capillary. The T_2 maps in Figure 3.16 are for the same biofilm sample but these velocity images are from the center slice while the T_2 maps are against a wall.

The velocity images in Figure 3.17 were taken at a flow rate of 8.0 cm/s in the axial direction which is the flow rate used to grow biofilms in the CBE laboratory. Although still laminar, this flow rate is more than twice as fast as the velocities measured in the experiments from Figures 3.10-3.13 which were 2.8 cm/s in the axial direction. Since the flow rate was higher during the growth of the biofilm, the velocity images shown in Figure 3.17 suffer from a misregistration artifact in which the velocity at a location in the system is registered at a pixel downstream in the image. This results in an artifact that produces the “arrowhead” shape of the non-axial v_x and v_y velocity components which does not affect the data in Figures 3.10-3.13. In Figure 3.17 the time between images is less than the interval for the T_2 maps in Figure 3.16. Note that the change in biofilm from 24 to 32 hours in Figure 3.16 is apparent but subtle. In contrast, the initiation of the secondary flow components, v_x and v_y , is significant. This indicates the strong dependence of the transport processes in a bioreactor on even small changes in biomass and the ability to use NMR measurements of fluid dynamics as a biosensor.

Propagators

The propagator is a function which describes the probability that a particle or spin has displaced a distance, Z , in a designated time, Δ , where $Z = z(\Delta) - z(0)$ for small times. Propagators were collected using NMR PGSE experiments for a clean and biofilm fouled capillary to compare with the theoretical velocity histogram generated with MatLab shown in Figure 3.18. The propagator is directly related to velocity in that velocity is a displacement over time, or Z/Δ . An observation time of $\Delta = 15$ ms was used for all three cases.

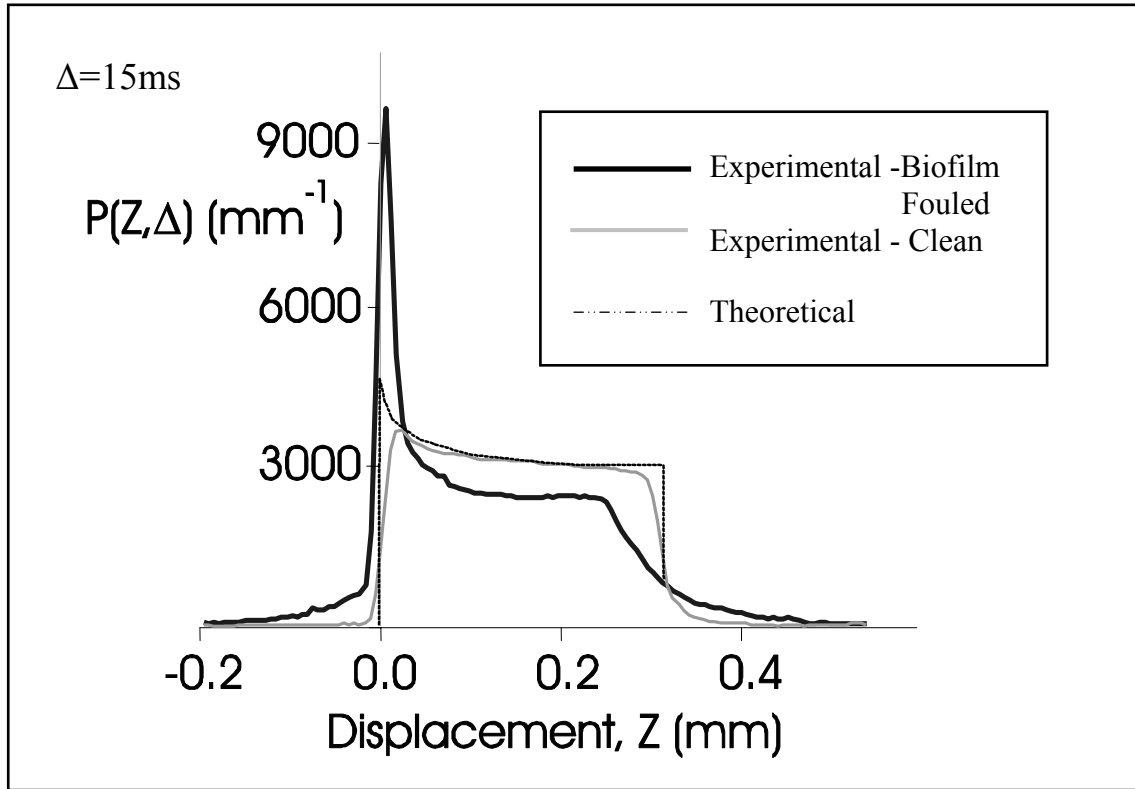


Figure 3.18. Propagator Data Comparison With $\Delta=15\text{ms}$. The propagator for the theoretical MatLab solution for a clean capillary is a box function with a small peak on the zero end which represents the zero and low velocities found in the corners of the square capillary. The experimentally measured propagator is in very good agreement with the theoretical profile, although it does display some diffusive broadening not seen in theory. The propagator for a biofilm fouled capillary displays a much larger peak at zero which is indicative of water bound up and immobilized within the biofilm. Figure reprinted from Seymour et al. [5].

The theoretical velocity distribution displayed above is very similar to that for a round tube where every velocity has an equal probability of occurring. The only difference in a square capillary is that there are areas in the corners of the capillary where there is slow or zero flow which is shown in the peak close to zero in the theoretical propagator. The experimentally measured propagator also displays this peak in the slow velocity region, in accordance with theory. The only difference between the theoretical and experimental measurement is in the diffusive broadening at each point of the graph

resulting in softened corners and small tails on the ends. This occurs because the molecules diffuse in the x- and y- (non-axial) directions during the observation time, Δ , thus randomly sampling different velocities [31].

The propagator for a biofilm capillary displays some significant differences from a clean capillary. Most noticeable is the large peak at zero indicating that there is a higher probability for a zero and very small displacements. This area corresponds to water that is restricted within biofilm and, therefore, undergoes diffusive motion. The biofilm also induces a larger probability of negative axial flows most likely found within the biofilm or at the biofilm, bulk fluid interface. This result is in agreement with the imaging data and an interesting result of this work which requires further theoretical and experimental study. There is also the development of a tail at high displacement region since the bulk fluid must move faster, *i.e.* displace further, to compensate for the smaller diameter available for flow due to the biofilm growth.

CONCLUSIONS

- Velocity maps constructed from NMR experiments for a clean, square capillary are consistent with the theoretical velocity profile.
- NMR microscopy techniques produce images of the overall structure of *Staphylococcus epidermidis* biofilms. Although much higher resolution can be obtained with other methods, such as confocal laser microscopy and scanning electron microscopy, NMR provides three-dimensional images of the entire biofilm, from substratum to fluid interface. NMR techniques are also non-destructive and able to acquire images without disrupting or altering the biofilm.
- Biofilm growth within a capillary significantly impacts the fluid dynamics. Axial velocity components become non-uniform and anti-symmetric as a result of flowing around clusters of biomass. In addition, secondary components of velocity, which are not found in clean capillaries, are induced in a capillary fouled by biofilm. These secondary flows display a distinct periodicity and are coupled in a way that indicates a helical secondary flow pattern. This result is highly significant and should change how mass transfer coefficient modeling of transport from bulk fluid to biofilm is performed by incorporating the enhanced convective mixing due to the secondary flow

components into mass transfer coefficient prediction and numerical simulations.

- Since NMR is a non-destructive technique, images of biofilms and the velocity patterns they produce over time can be imaged while the biofilm is growing. This provides the potential to acquire data which can be used to develop growth dependent transport models.
- Propagators for biofilm fouled capillaries display probabilities for velocities higher than in clean capillaries. This is due to the flow being forced to speed up as the capillary diameter decreases from the formation of biofilm which does not allow for bulk flow. The propagators also display an increased probability of zero displacement because of the spins within the biofilm being bound up and unable to move.

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APPENDICES

APPENDIX A

SCANNING ELECTRON MICROSCOPY IMAGES

Introduction

Scanning Electron Microscopy (SEM) is an imaging technique that provides micron-scale resolution that is ideal for studying bacteria. One of the drawbacks of the technique is that the sample must be dehydrated then coated with gold. This process often destroys overall structure because the biofilm collapses as a result of the EPS being dehydrated. SEM imaging is in stark contrast to NMR imaging where the resolution is on the order of tens of microns and no physical manipulation of the sample is required to acquire images.

Procedure

A *Staphylococcus epidermidis* biofilm was grown for 48 hours in a one-millimeter, square, glass capillary using the same setup shown in Figure 2.1 (page 17). The biofilm fouled capillary was then filled with a water solution containing 10% ethanol by volume. The sample was allowed to sit in this solution for 20 minutes then a 25% ethanol solution was injected. The procedure was then repeated with the following ethanol strengths: 40%, 50%, 70%, 80%, 95%, and 100%. After the dehydration process the sample was broken into fragments to expose the biofilm sample. A fragment was chosen by naked eye inspection based on the amount of biomass that was exposed. The fragment was then given to Rick Veeh of the Imaging and Chemical Analysis Laboratory (ICAL) in the Center for Biofilm Engineering to perform the SEM Imaging. The sample was coated with gold and placed inside the SEM microscope.

Results and Discussion

The SEM images in Figures A.1 and A.2 display the high level of resolution that is one of the hallmark features of SEM imaging. Individual bacterial cells within the biofilm can be easily distinguished with the technique and the number and density of cells within a biofilm cluster becomes apparent. One downfall of the technique is the dehydration process which collapses the structure of the biofilm leaving only a skeleton of the biomass. The EPS, which is the building material of the biofilm, is barely visible and can only be seen as small strands stretching across the cells. This technique for imaging biofilms, in sharp contrast to NMR imaging, is destructive to the sample.

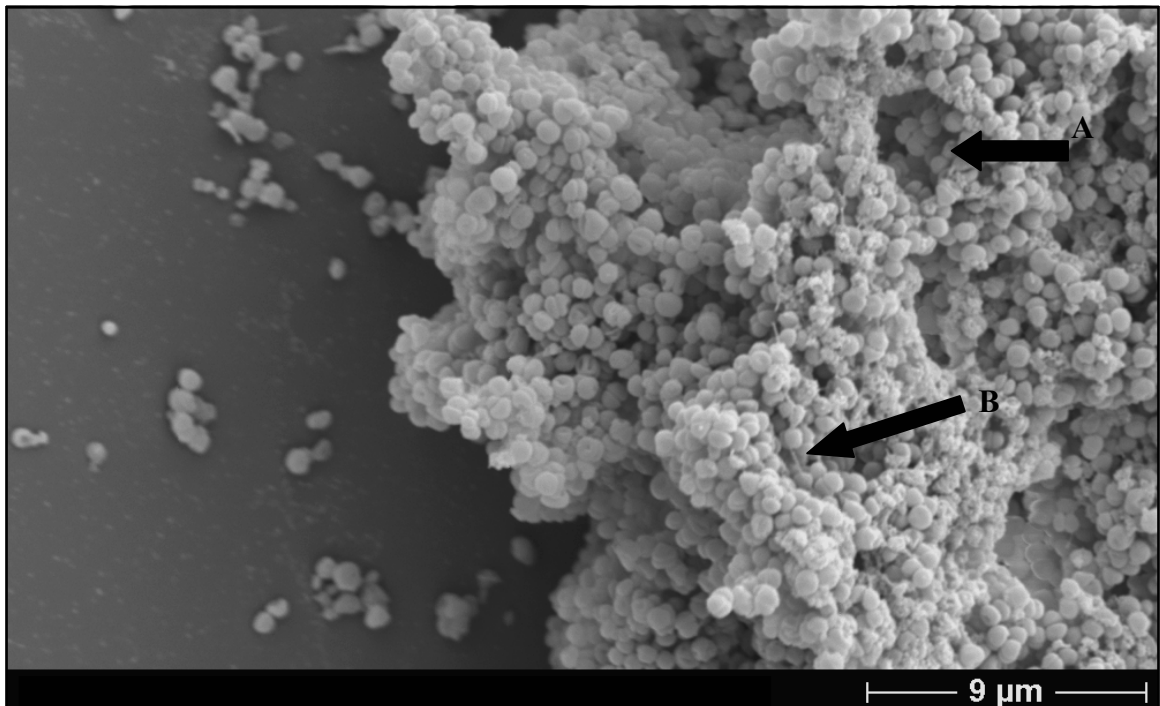


Figure A.1. SEM Image. This SEM displays (A) the large channels that are formed in a biofilm and (B) strands of dehydrated EPS.

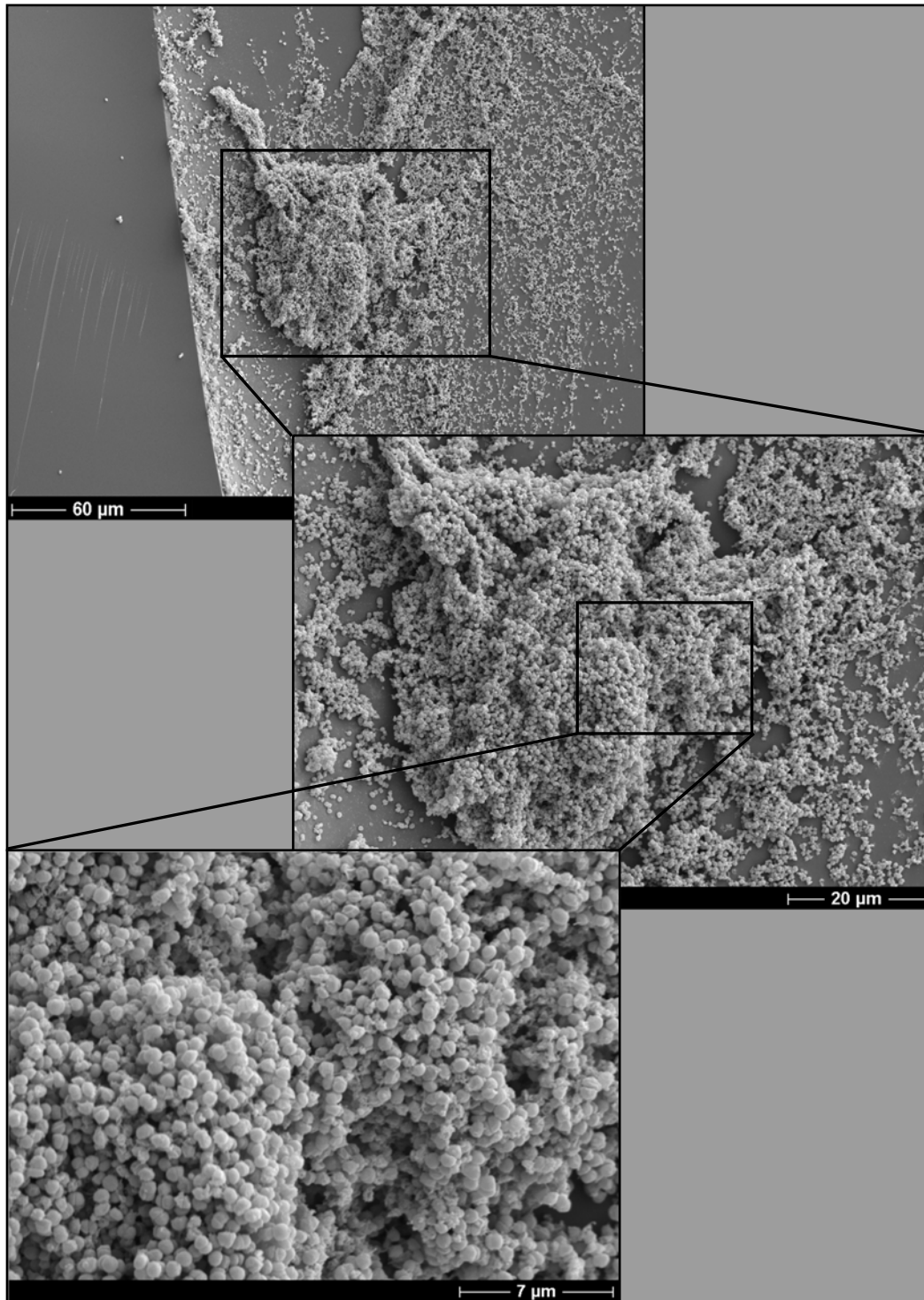


Figure A.2. Resolutions of SEM. This figure displays three different resolutions of a biofilm cluster.

APPENDIX B

NMR SPECTROSCOPY OF BIOMASS

Introduction

NMR spectroscopy is one of the best techniques for determining the chemical composition of a sample. The work presented here shows the chemical spectra obtained from a concentrated *S. epidermidis* biofilm.

Procedure

To obtain a chemical spectra of the biomass a different technique for growing the biofilms was used. Instead of being grown inside a glass capillary, the biofilm colonies were grown on membranes on top of agar plates. The bacterial strain used was *Staphylococcus epidermidis* and the growth medium was Tryptic Soy Agar (TSA). The membranes were UV sterilized on each side for 20 minutes then placed on the agar plates. The membranes were inoculated with 10 μ L of suspended bacterial solution with an absorbance of 0.035 relative to sterile TSB. Five membranes were placed on each plate and six plates were used totaling 30 biofilm colonies. The membranes were transferred to fresh agar plates after 24 hours and harvested after 48 hours.

To harvest the biomass the membranes are placed inside a dilution tube with approximately 20 mL of buffer solution and then vigorously agitated on a desktop vortexer to detach the biomass from the membranes. The buffer solution containing the biomass was then centrifuged to separate the water from the biofilm. The water was then pulled off the top using a syringe and the biofilm sample was scrapped off the bottom of the tube and placed in an NMR tube. It should be noted that it was very difficult to transfer the concentrated biomass into the NMR tubes since it is so sticky. In the future the samples should be centrifuged in the NMR tube directly and then the water removed.

The concentrated biofilm sample was then taken to the Chemistry Department where Dr. Scott Busse performed the NMR spectroscopy.

Results and Discussion

The NMR spectra of condensed biomass are shown for both 250 MHz (Figure A.3) and 500 MHz (Figure A.4). The spectra at the two different magnet strengths display nearly the same resolution of peaks. Since the 250 MHz magnet is used for NMR imaging experiments this result opened up the possibility of doing NMR spectra in combination with imaging experiments.

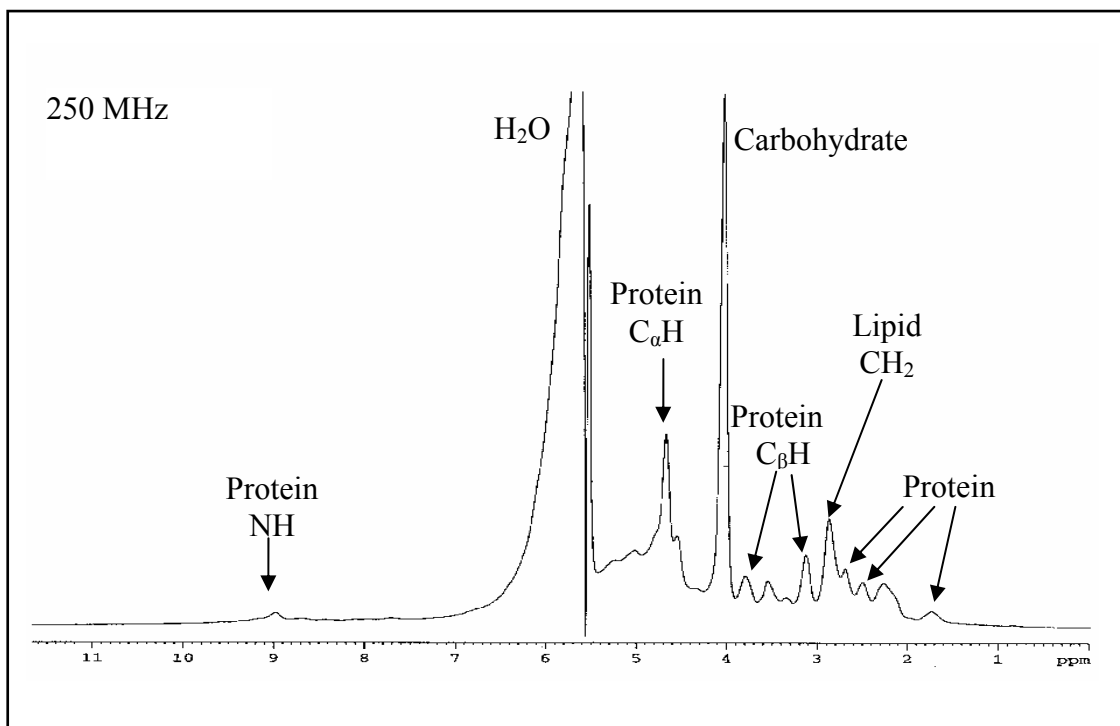


Figure A.3. NMR Spectra at 250 MHz. The 250 MHz magnet is employed for NMR imaging experiments and it used here to acquire traditional NMR spectra.

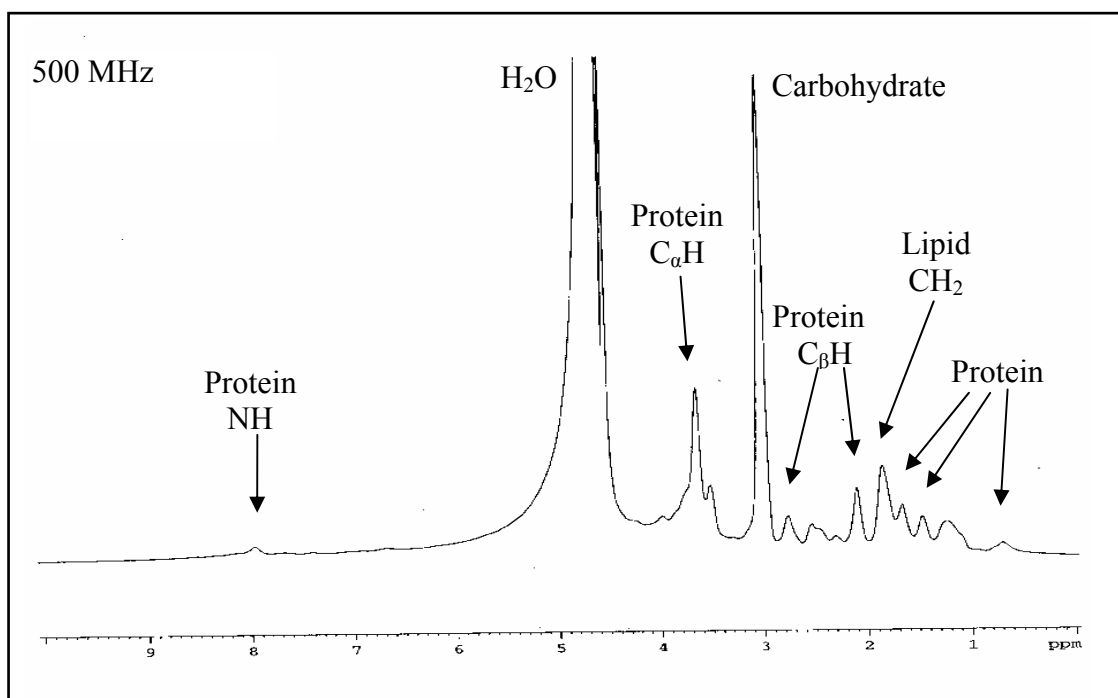


Figure A.4. NMR Spectra at 500 MHz. Both the 250 and 500 MHz display sufficient resolution to distinguish the various peaks from a sample of condensed biofilm.

APPENDIX C

3 MM CAPILLARY

Introduction

The periodicity of the secondary flows induced by biofilm formation seems to be on the same scale as the diameter of the capillary (1 mm). In order to investigate this possible scale dependence, experiments were performed with a 3 mm capillary since they were readily available in the laboratory.

Procedure

The first experiments were conducted with a clean 3 mm capillary to determine what experimental parameters to use. The experimental setup was the same as for the 1 mm capillary except that a pump was needed in order to produce flow rates large enough to yield the same Reynolds number (flow rate=172 mL/hr, $Re=16$) for the 1 mm capillary. The parameters that produced the best velocity images were the same as for the 1 mm except the TR had to be increased to 4000 ms which significantly increased the experiment acquisition time by a factor of 4 to get the same resolution. Experiments with a biofilm fouled capillary were grown first in the incubator with a pump then taken to the NMR lab to perform NMR experiments using the same parameters as for the clean capillary but using the 8 mm rf coil.

Results and Discussion

Velocity images of a clean, 3 mm capillary are shown in Figure A.5. These images show that the velocity profile is identical to that for the clean 1 mm capillary, as expected. Images of a biofilm fouled capillary were never completed due to the fact that the biofilm growth was not the same in the larger capillary and the biofilm would slough

off within a few hours of being placed vertically inside the NMR magnet. Figure A.6 displays an image captured before sloughing that seems to suggest that secondary flow patterns were induced on a scale of the order of the capillary cross section. However, a full characterization was never completed before the biofilm would slough off.

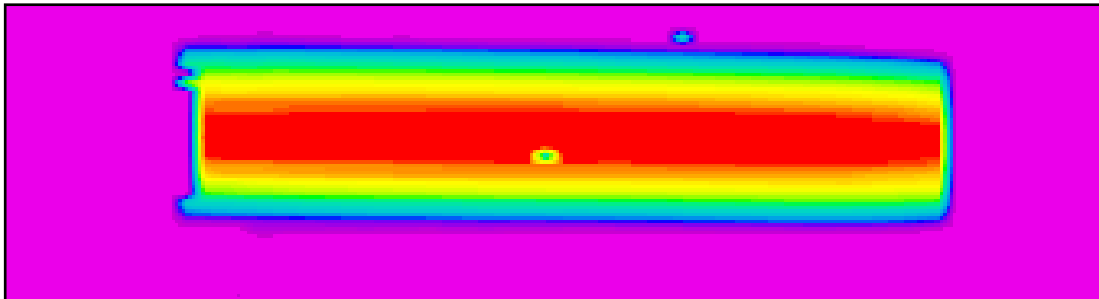


Figure A.5. Clean 3 mm Capillary. The z-direction velocity map for a clean 3 mm capillary displays the same smooth, uniform, and symmetrical profile as a 1 mm capillary.

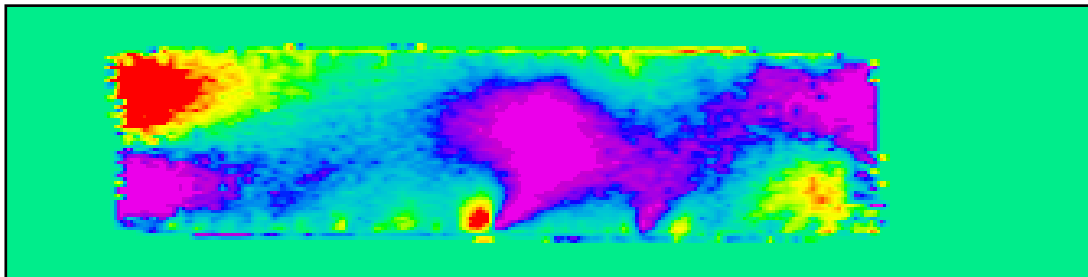


Figure A.6. Biofilm Fouled 3 mm Capillary. The phase (x) direction velocity map of a biofilm fouled capillary seems to display similar features as those seen in a 1 mm capillary. A full and repeatable characterization of a biofilm fouled capillary was never accomplished. The z-component of velocity for this sample was the same as Figure A.5 and no perturbations were seen.

APPENDIX D

CTC DYE EXPERIMENTS

Introduction

CTC (5-cyano-2,3-ditolyl tetrazolium chloride) is a dye that produces formazan, in the form of crystals, when it is reduced within a cell. It is currently used as an indicator of respiratory activity because the reduced CTC is deposited and fluoresces at 350 nm in confocal microscopy. Since the CTC turns into a crystal structure when it is reduced, NMR parameters such as T_1 , T_2 , or proton density should be different for areas stained with CTC and those within the biomass and free water. The contrast produced by these differing relaxation times should produce an image which displays the zone of active respiration within a biofilm. This concept is the start of an attempt to use NMR to study the relationship between structure within a biofilm and the function of the cells.

Procedure

Since this work was purely exploratory, a biofilm sample was grown up and a full battery of NMR images and velocity maps were collected before the CTC dye experiment. The CTC dye was then injected into the capillary containing the biofilm sample. Mobile proton density and T_2 maps of the sample were immediately taken and continued to be acquired for the next 3 to 4 hours. The technical data sheet for CTC dye states that the staining can take anywhere from 30 minutes to 2 hours. The time generally used is 2 hours in order to ensure that the biofilm is fully stained. The images were taken every 20 minutes in hopes of seeing the development of the active layer as the biofilm reacts with the CTC dye.

Results and Discussion

Figure A.7 displays the results of the CTC dye experiments. The mobile proton density map prove to be the best indicator where the CTC dye crystals were being deposited since the protons within the crystals where either restricted or excluded by the crystal structure compared to that within the biofilm.

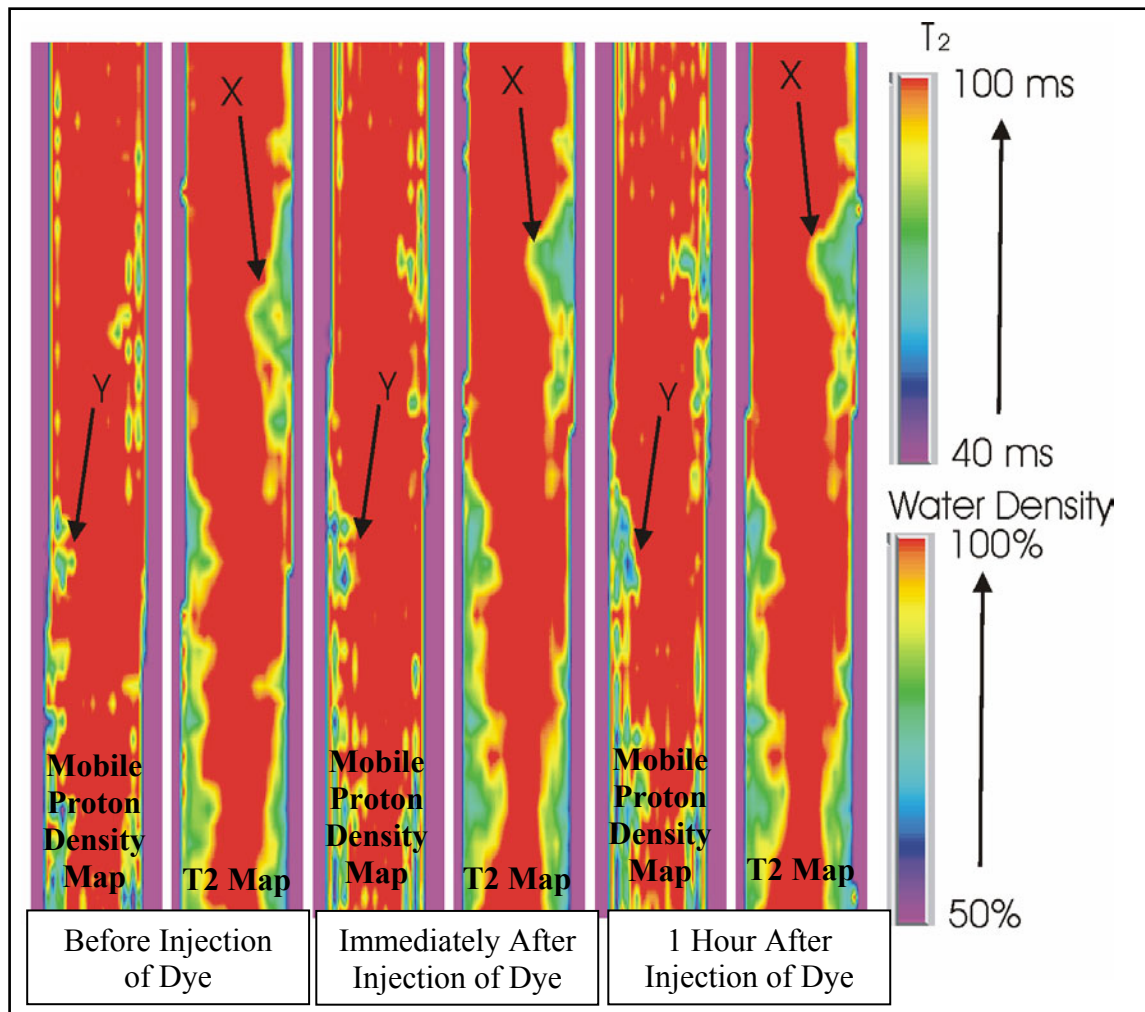


Figure A.7. CTC Dye Experiments. The mobile proton density maps showed the best contrast of where the CTC dye was interacting with the biofilm as seen at point Y. T_2 maps did not show any difference from before and after the injection of dye other than a redistribution of the mass of biofilm shown at point X because of the change in fluid flow direction.