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IMAGING OF A MODEL PLASTIC FAT SYSTEM BY 3-DIMENSIONAL
WIDE-FIELD TRANSMITTED POLARIZED LIGHT MICROSCOPY AND
IMAGE DECONVOLUTION

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

JERROLD W. LITWINENKO

In partial fulfilment of requirements

for the degree of

Master of Science

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ABSTRACT

IMAGING OF A MODEL PLASTIC FAT SYSTEM BY 3-DIMENSIONAL WIDE-FIELD TRANSMITTED POLARIZED LIGHT MICROSCOPY AND IMAGE DECONVOLUTION

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This research emphasizes the development of a novel technique for 3-dimensional imaging of fat crystal networks. A model fat system was crystallized by conventional methods (a 2D thin film of 20 μm in thickness) and in a volume of approximately 200 μm in thickness. 3-Dimensional data sets of optical slices through the volume were then deconvolved by a nearest neighbours (NN) algorithm and a blind algorithm. NN deconvolution was not successful in removing out-of-focus pixels, however blind deconvolution was highly successful, as was demonstrated by 3D renderings and 2D projections of the volumes. Blind deconvolved images of slices and 2D projections of the volume were analysed and compared to results for conventional thin film crystallization. Image analysis of the volume slices and projections had a lower area fraction, lower particle counting fractal dimension (D_f), and smaller mean particles sizes, indicating that confinement effects in thin films results in more orderly crystallization. The fractal dimension D_b , on the other hand, was not sensitive to confinement. Fractal dimension retention upon projection of 3D slices onto a 2D plane provided novel experimental evidence for this theorized behaviour. Constancy in fractal dimension values was demonstrated under variable imaging criteria including microscopy optics, objective magnification, type of camera, and image size and resolution. A novel means of determining the relative degree of order in particle distributions by analysis of nearest neighbour distances was also successfully demonstrated.

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E. LIST OF ABBREVIATIONS USED

Φ	solids' volume fraction
ξ	average floc size
$^{\circ}$	degrees
$^{\circ}\text{C}$	degrees Celsius
$^{\circ}\text{C}/\text{min}$	degrees Celsius per minute
2D	2-dimensional
3D	3-dimensional
4D	4-dimensional
a	primary particle size
A	Hamacker's constant
AFM	atomic force microscopy
CCD	charge coupled device
COSM	computational optical sectioning microscopy
CSLM	confocal scanning laser microscopy
D	fractal dimension
d	Euclidean dimension
d_o	inter-particle distance
Db	box-counting fractal dimension
Df	particle-counting fractal dimension
DLA	diffusion-limited aggregation
DVD	digital video disc
Dy	dynamic (shear present)
EM	electron microscopy
FFT	fast Fourier transform
G'	shear elastic modulus
GHz	gigahertz
h	hours
HMF	high-melting fraction of milk fat
IPTK	image processing toolkit
kb	kilobytes
K	elastic constant
L	length
Mb	megabytes
MES	microstructural element size
MLE	maximum likelihood estimation
N	number of particles
NIH	National Institute of Health
nm	nanometres
NN	nearest neighbours (deconvolution)
NND	nearest neighbour distance
PC	personal computer (Windows OS)
PLM	polarized light microscopy

PSF	point spread function
R	Pearson linear correlation coefficient
RAM	random access memory
RLCA	reaction-limited cluster aggregation
ROI	region of interest
St	static (no shear present)
SEM	scanning electron microscopy
SFC	solid fat content
TEM	transmission electron microscopy
TLB	transmitted light brightfield
UK	United Kingdom
w/w	weight to weight proportion
x	backbone fractal dimension
X	times (magnification)
μL	microliters
μm	micrometers

1. BACKGROUND

1.1. *Introduction*

Food lipids such as butter, margarine, cocoa butter, vegetable shortenings, and lard are widely used as functional ingredients in foods high in fat (de Man, 1961; Heertje et al., 1988; Shukla & Rizvi, 1996; Narine & Marangoni, 1999a; Campos et al., 2002; Campos et al., 2003; Litwinenko et al., 2002; Marangoni & McGauley, 2003). The primary role of fats in foods is to impart sensory attributes such as creaminess, mouthfeel, spreadability, melting profile, cohesiveness, and structure (Shukla & Rizvi, 1996; Sato, 1999; Narine & Marangoni, 1999a; Wright et al., 2000; Sato, 2001; Litwinenko et al., 2002; Marangoni, 2002). In many food applications, the fat component exists in a semi-solid state, in the form of a fat crystal network (Narine & Marangoni, 1999a,b,c; Marangoni, 2002).

Since fats are composed of a wide variety of triglycerides (i.e., they are multi-component systems) they exhibit a wide range of melting and crystallization temperatures. This property of fats results in a high degree of variability of crystallization behaviour when subjected to various crystallization conditions. Variables such as the rate of cooling, the degree of supercooling, the presence of shear and storage time can all result in different microstructures (de Man, 1961; de Man, 1964; Heertje et al., 1988; Grall & Hartel, 1992; Kellens et al., 1992; Heertje, 1993; Fairley et al., 1995; Kawanari, 1996; Blaurock, 1999; Narine & Marangoni 1999c; Herrera & Hartel, 2000; Wright et al., 2000; Wright & Marangoni 2002; Campos et al., 2002; Litwinenko et al., 2002; Marangoni & McGauley, 2003). Altering crystallization conditions results in differences in the properties of the network on the microstructural level which can include changes in crystal size, morphology,

number, and their degree of distributional order (Narine & Marangoni 1999c; Litwinenko et al., 2002; Marangoni & McGauley, 2003). These changes on the microscopic level result in changes in the interactions between the particles that make up the network, which in turn influence macroscopic properties (Narine & Marangoni 1999a,b,c; Marangoni 2000; Marangoni 2002). It is therefore important to further our understanding of the relationship between microstructure and rheological properties.

Of particular interest is the fractal scaling behaviour demonstrated by fat crystal networks. 2-Dimensional fractal analyses on images of thin film networks have been extensively studied, and suggest that the relative degree of order and fill in the networks can be quantified, and used to make predictions pertaining to mechanisms of network formation and macroscopic rheological properties (Narine & Marangoni 1999a,b,c; Marangoni 2000; Marangoni 2002; Litwinenko et al., 2002). These analyses have been compared to 3-dimensional fractal dimensions determined by rheological methods with intermediate success (Narine & Marangoni 1999a; Litwinenko et al., 2002; Marangoni, 2002). This has led to many questions related to the fundamental differences between 2D and 3D crystallization conditions and their resulting fractal dimensions. Imaging and fractal analysis of fat crystal networks in 3D would therefore be of high value.

Imaging of fat crystal networks in 3-dimensions, however, is far from trivial. Many efforts have involved 3D imaging techniques such as confocal, electron, and atomic force microscopies and have resulted in successful but limited interpretations of the 3D structure of bulk fats (Manning & Dimick, 1985; Heertje et al., 1987; Heertje & Leunis, 1997). Although all of these techniques are highly valuable in their own respects, limitations inherent to these methodologies hinder the ability to resolve and view the internal structures of thick sections

or volumes of bulk fats. For this reason, a novel 3D imaging technique capable of viewing thick sections of bulk fats was pursued.

3-Dimensional imaging using fluorescence and brightfield light microscopies, and confocal laser scanning microscopy has been well established (Abbott & Nugent, 1992; Krishnamurthi et al., 1992; Stevens, 1994; Ancin et al., 1996; Barta et al., 1997; McNally et al., 1998; Wallace et al., 2001; Verbeek & Boon, 2002; Verveer et al., 2002). Optical sections through a volume are acquired by using a z-stage motor, and contain both in and out-of-focus detail. The process of image deconvolution reassigns unfocussed pixels to their most likely location by means of complex 3-dimensional algorithms (McNally et al., 1999; Wallace et al., 2001). The main objective of this study was to adapt and assess the validity of this optical sectioning 3D imaging and deconvolution technique to a transmitted polarized light imaging modality. Upon doing so, a new means of observing fat crystal networks in unconfined space would be established, and a greater understanding of the effects of confinement on network formation and fractal dimensionality could be pursued.

1.2. Objectives

The overall aims of this study were as follows:

- 1) To develop and assess the validity of a new 3D imaging technique. This involved the design of a sample holder that would allow for volumetric imaging of fat crystal networks. 3D volume data sets were then obtained by wide-field polarized light microscopy. These data sets were then to be deconvolved by two different methods (nearest neighbours and blind algorithms) and their relative successes compared.
- 2) To further our understanding of the crystallization of fats in 3-dimensions and make comparisons to the results of traditional 2-dimensional thin film crystallization conditions. This included an examination of crystal morphology, particle sizes and distributions of mass in both volume and thin film conditions to investigate possible mechanisms or network formation and influences of confinement on network properties and growth.
- 3) To further our understanding of the fractal nature of fat crystal networks. This involved the determination of fractal dimensions of 2D slices from a 3D volume, as well as 2D projections of a 3D volume. Also, the development of a novel means of determining a relative degree of order from images was pursued. These studies aimed to further our understanding of the factors that influence fractal dimensions

determined by particle-counting and by box-counting methods (D_f and D_b) as well as comparison to those determined from 3D rheological methods.

- 4) To offer insight into future developments of this new 3D imaging technique. This included an investigation into the assumptions and limitations inherent to the technique being developed. Suggestions for further experiments and verifications of the validity of the new method were also an aim of this research.
- 5) To determine if changing imaging systems has any impact on image analysis fractal dimension parameters D_f and D_b . This involved comparison of results from this study to results obtained in the past, using an older microscope and camera system.

1.3. Literature Review

1.3.1. The Fractal Nature of Fat Crystal Networks

The Concept of Fractality

Fractal geometry was first proposed by Benoit Mandelbrot in 1982 as a means of quantifying objects of complex structure that defy classical Euclidean geometry (Mandelbrot, 1982).

In order for an object or distribution of objects to be fractal in nature, the following conditions must be met:

- It must have a dimensionality of non-integer (non-Euclidian) value
- It must display self-similarity
- It must exhibit scaling behaviour within the range examined

The concept of fractality is difficult to understand without first referring to a few examples that demonstrate the concept.

Classical Euclidean geometry deals with objects that possess dimensionalities of integer values. These objects fill Euclidean space or their “embedding” space of dimension d . Examples that fall into this category include a line ($d=1$) a plane ($d=2$) and a cube ($d=3$) (Narine and Marangoni, 1999a). Fractal geometry, on the other hand, serves as a means to quantify objects of intermediate, or non-integer values, and are assigned a fractal dimension D (Viscek, 1992). For example, if kinks were introduced in a line or plane, or an object of approximate cubic shape contained voids, these objects would no longer exist as classical Euclidean objects with integer dimensions (Narine and Marangoni, 1999a).

Self-Similarity and Scaling Behaviour

Perfect fractal objects are objects that display scale invariance (i.e. they display exact self-similarity). This put simply means that fractal objects appear the same at various scales (Viscek, 1992). Natural systems, however, do not occur as perfect fractals, but rather display statistical self-similarity within a finite range of scales since randomness is introduced by the complexity of structure formation (Narine and Marangoni, 1999a, Marangoni, 2002). A common example of statistical self-similarity is the tree analogy: a small branch of a tree closely resembles a large branch of the tree, which closely resembles the tree. Although (by comparison to perfect fractals) the range of fractal scaling behaviour exhibited by natural systems is narrow, this does not mean that natural systems are not fractal (Marangoni, 2002). For example, it has been demonstrated in colloidal gels that scaling behaviour exists within the range the size of primary particles to the size of the clusters of particles (Shih et al., 1990; Marangoni & Hartel 1998; Narine & Marangoni 1999a; Martinez-Lopez et al., 2001; Marangoni, 2002). Knowledge of a materials structure allows for fractal analyses to be performed within the range of scaling behaviour (Marangoni, 2002).

Fractal Dimension Determination

Fractal dimension determination is often demonstrated with a classical example. If one were to closely examine a map of the coast of England, which is approximately a plane ($d=2$) by iteratively measuring its perimeter with rulers of decreasing size it's fractal dimensionality ($D<2$) could be determined (Viscek, 1992). This is an example of the fractality of an object determined by the scaling relationship between perimeter and length, and is just one of many methods that can be used to determine an objects fractal dimension.

Determination of fractal dimensions of an object or distribution of objects in space can also be performed by examining mass-distance relations (Barnsley 1988; Viscek, 1992; Solecka-Cermakova & Vlckova, 1996; Rothchild, 1998; Narine & Marangoni, 1999a). Typical mass-radius techniques involve the iterative computation of a property of the object (which can be mass, area, length, density, etc.) as a function of decreasing radius of a circle placed on the object (Rothchild, 1998). The general relationship examined by mass-distance techniques is as follows:

$$P(R) \approx R^D \quad (1.1)$$

where the measured property (P) is proportional to the radius of the circle (R) containing the measured property raised to the exponent D, which is the objects fractal dimension. Plotting $P(R)$ as a function of R in a log-log fashion results in a linear relationship with a slope equal to D (Barnsley 1988; Viscek, 1992; Rothchild, 1998). Mass distance methods are usually preformed on objects in 2-dimensional embedding space, or projections of 3-dimensional objects onto 2-dimensional space. This is the case when examining fractal dimensions from images of microstructures formed in thin films, and 2D projections of 3D volumes, respectively.

Additional methods for fractal dimension determination involve the scaling behaviour exhibited by physical properties of fractal systems (Shih et al., 1990; Liu et al., 1990; Wessel & Ball, 1992; Haw et al., 1997; Krall & Weitz, 1998; Gonzalez et al., 1999; Tather & Erdem-Senatalar, 1999; Cipelletti et al., 2000). One pertinent example is the 3-dimensional scaling behaviour observed between mechanical properties and solids volume fraction, and will be

discussed in more detail to follow. Before referring to this specific example, however, a short discussion on the mechanisms of fractal network formation is appropriate.

Diffusion-Limited Aggregation (DLA)

The rate of growth of larger structures that make up fractal networks (including colloidal gels and fat crystal networks) is believed to be dictated by heat and mass transfer (diffusional) processes (Rothchild, 1998; Narine & Marangoni, 1999a). In the case of fat crystal networks, primary particles (~2-6 μm in size) formed by nucleation and growth diffuse to the surface of other primary particles and aggregate to form larger microstructures (~100-200 μm) which then aggregate to form a solid 3-dimensional network (Narine & Marangoni, 1999a). This theory suggests that network formation may be due to both particle-cluster and cluster-cluster aggregation processes, which may confound the fractal dimension determination (Viscek, 1992). Computer simulations have been performed to predict the fractal dimensions of structures formed by various diffusional mechanisms including particle-cluster aggregation and cluster-cluster aggregation (Barnsley, 1988; Viscek, 1992; Rothchild, 1998).

Particle-Cluster Mechanism

In the simplest of cases, DLA occurs when drifting particles irreversibly adhere to existing clusters, leading to a branched, wispy structure with $D=1.7$ in 2D embedding space and $D=2.5$ in 3D embedding space (Viscek, 1992; Rothchild, 1998).

Cluster-Cluster Mechanism

In more complicated cases of DLA, rather than particles diffusing and adhering to existing clusters, a process of irreversible cluster-cluster aggregation occurs leading to the formation of single large clusters. In this instance, computer simulations result in a predicted fractal dimension $D=1.7-1.8$ in 3D embedding space (Rothchild, 1998).

Reaction-Limited Cluster-Cluster Aggregation (RLCA)

As is implied by its name, for RLCA the rate of aggregation depends not on diffusional processes (as in DLA), but rather on the reaction rate of cluster-cluster aggregation. This implies that when clusters come together they may be reversibly unattached (Viscek, 1992; Rothchild, 1998). It is therefore highly unlikely that fractal analysis of irreversible colloidal gels or fat crystal networks would reveal RLCA behaviour. Computer simulations predict a mass fractal dimension of $D=1.53$ and $D=1.98-2.11$ for reaction-limited growth in 2D and 3D embedding spaces respectively (Viscek, 1992; Rothchild, 1998).

Rules of Fractal Scaling

As will be discussed later, efforts to determine the fractal dimension of fat crystal networks have been performed by both 2D and 3D analyses (image analysis of thin films and rheological scaling methods respectively) and their values compared (Narine & Marangoni 1999a; Litwinenko et al., 2002; Marangoni 2002). This being the case, there are a number of “rules” related to fractal scaling that are relevant to our studies. In particular, rules related to

fractal dimensions in varying Euclidean spaces are of interest. Viscek states the following (Viscek, 1992):

- 1) Many times it is the projection of a fractal which is of interest or can be experimentally studied (e.g., a picture of a fractal embedded into $d=3$). In general, when projecting a $D < d-m$ dimensional fractal onto a $d-m$ dimensional surface results in a structure with the same fractal dimension $D_p = D$. For $D \geq d-m$ the projection fills the surface, $D_p = d-m$.
- 2) Cutting out a $d-m$ dimensional slice (cross section) of a D dimensional fractal embedded into a d dimensional space usually leads to a $D-m$ dimensional object.

Interpretation of rule 1 would suggest that a fractal object of dimensionality of $D < 2$ in 3D space, when projected onto a 2D plane, would retain its fractal dimension ($D < 2$). If on the other hand, the object in 3D embedding space had a fractal dimension of $2 \leq D \leq 3$ when projected onto a 2D plane, it would fill the plane and result in a $D=2$.

Rule 2 suggests that if a 2D cross section were taken of a fractal with dimensionality D embedded in 3D space its fractal dimension would be reduced to $D-1$. This rule has also been interpreted in reverse, with a value of 1 added to D when translating a fractal measured on a 2D plane into 3D space. Little in the way of experimental evidence can be found to verify the validity of such an interpretation, however in some cases this translation has been found to be satisfactory (Hagiwara et al., 1997; Litwinenko et al., 2002; Marangoni, 2002).

A clearer understanding of these complex fractal scaling principles, and their relevancy to our studies, can be attained following a discussion on how these principles were

first developed by Brown and Ball (Brown & Ball, 1985; Brown & Ball, 1987) and later demonstrated for the analysis of colloidal aggregates (Shih et al., 1990). For the sake of simplicity and relevancy, discussions on fractal scaling theory to follow will focus on the theory introduced by Brown and Ball and the techniques demonstrated by Shih et al., followed by their adaptation to fat crystal networks. Continuing efforts to determine the fractal dimension of fat crystal networks by image analysis of microstructure will also be discussed.

Fractality as it Pertains to Colloidal Gels

Brown and Ball demonstrated that colloidal aggregates behave as 3-dimensional mass fractals (Brown & Ball, 1985; Brown & Ball, 1987). In colloidal gels that are composed of a disordered distribution of mass that contain statistical self-similar patterns, the observed power-law relationship between the elastic modulus and the solid volume fraction was discovered (Brown & Ball, 1985; Brown & Ball, 1987). These findings formed the basis for continued studies by Shih et al. who developed a scaling theory that explained the elastic properties of colloidal gels via the fractal flocculation within two distinct rheological regimes: the weak-link and the strong-link (Shih et al., 1990).

The Strong-Link Regime

At low particle concentrations clusters grow large and act as weak springs, and as a result, the elastic constant of the gel (K) is dominated by the elasticity of the flocs themselves, and follows the relationship:

$$K \approx \phi^{(d+x)/(d-D)} \quad (1.2)$$

where Φ is the volume fraction, d the Euclidean dimension, D the fractal dimension, and x is the backbone fractal dimension (Shih et al., 1990). However, this relationship is does not apply to fat crystal networks since at low volume fractions a solid 3-dimensional network is not formed (Narine & Marangoni, 1999a).

The Weak-Link Regime

At high particle concentrations small flocs act as strong, hard springs, and as a result, the elastic constant of the gel (K) is dominated by the interfloc links. This can be expressed in a system of size L :

$$K \approx \left[\frac{L}{\xi} \right]^{d-2} K_l \approx \xi^{-(d-2)} \quad (1.3)$$

where K_l is the elastic constant of the interfloc links, and ξ is the average size of the flocs.

If the fractal dimension of the microstructures is D , and assuming that the volume fraction within flocs is equal to the volume fraction of the system (Shih et al., 1990; Witten, 1999) then the scaling relationship between the average floc size and the volume fraction can be expressed as:

$$\xi \approx \phi^{\frac{1}{(D-d)}} \quad (1.4)$$

Combining equations 1.2 and 1.3, it follows that:

$$K \approx \phi^{(d-2)/(d-D)} \quad (1.5)$$

and for an object in 3-dimensional Euclidean space:

$$K \approx \phi^{1/3-D} \quad (1.5)$$

Consequently, a log-log plot of the elastic constant (K) versus the volume fraction (Φ) is linear with the slope being $-D$. The value of D is the fractal dimension of the colloidal gel.

Since the initial discovery of the fractal nature of colloidal gels, much research has verified the validity of the model. In 1992, Wessel and Ball used fractal scaling properties to examine fractal aggregation in gels under applied shear (Wessel & Ball, 1992). Colloidal aggregation experiments employing light scattering techniques have been successful in assessing cluster structure and the arrangement of clusters in gels (Haw et al., 1997) and in determining internal dynamics and elastic moduli of very weak gels (Krall & Weitz, 1998). Recent work has also examined the concentration dependence of the cluster fractal dimension in colloidal aggregation (Gonzalez et al., 1999).

The Adaptation of Fractal Scaling to Fat Crystal Networks

Vreeker et al. were the first to suggest that the fractal scaling behaviour observed in whey protein gels could hold true for fat crystal networks since the structure of fat crystal networks resembles closely that of a flocculated colloidal system (Vreeker et al., 1992a,b). In 1996, Rousseau and Marangoni applied the model developed by Shi et al. to fat crystal networks at high volume fractions (in the weak-link regime) (Rousseau & Marangoni, 1996a). They found that the shear elastic modulus of fat systems varied with the solid volume fraction as a power-law relationship, much like had been observed previously in colloidal gels. Since microstructures are the closest level of structure to the macroscopic network, it stood to reason that the application of small deformations on the network could be related to the fractal dimension as adapted from the model proposed by Shih et al. (equation 1.5) as follows:

$$G' \approx \lambda \phi^{1/(3-D)} \quad (1.6)$$

where G' is the shear elastic modulus determined within the linear viscoelastic region, Φ is the volume fraction (solid fat content/100), D is the fractal dimension, and

$$\lambda = \frac{A}{\pi a d_o^2 \gamma_o} \quad (1.7)$$

where A corresponds to Hamaker's constant, a to the diameter of the primary particles, d_o to the inter-cluster separation distance, and γ_o to the strain at the limit of linearity (Narine &

Marangoni, 1999a,b,c, Marangoni, 2002, Marangoni & Rogers, 2003). It must be noted, however, that the value of λ only remains constant within a certain range of solids' volume fraction. At higher volume fractions and following the formation of the network structure, coarsening may affect the value of λ (Marangoni, 2002). A representative schematic of a fat crystal network is presented in Figure 1.1.

Figure 1.1: An idealized schematic of a fat crystal network with an applied stress.

Weak Link Rheological Method for the 3-Dimensional Fractal Dimension Determination

To determine an estimate of the 3-dimensional fractal dimension of fat crystal networks the shear elastic modulus is measured at varying volume fractions (Rousseau & Marangoni, 1996a; Narine & Marangoni, 1999a,b,c; Marangoni, 2002). This is performed by creating dilutions of the material in a solvent that does not solubilize the material in the temperature range at which rheological measurements are to be performed (Narine & Marangoni, 1999a,b,c; Marangoni, 2002). This has typically been performed by diluting the fat system in canola oil and crystallizing the dilutions at 5°C (Narine & Marangoni, 1999a,b,c; Marangoni, 2002). The slope of the linear relationship between the storage modulus (G') and volume fraction (SFC/100) determines the fractal dimension of the fat

system (Narine & Marangoni, 1999a,b,c; Marangoni, 2002). A representative plot depicting results for lard is presented in Figure 1.2.

Since self-similarity is observed in polarized light images of fat crystal networks, many efforts to determine the fractal dimensions of fat systems on the basis of microstructure have also been pursued and will be addressed in the following section.

Figure 1.2: Log-log plot of the storage modulus (G') versus the solids volume fraction (solid fat content/100) used to determine the 3-dimensional fractal dimension of a lard crystal network (adapted with permission from Marangoni, 2002).

Fractal Dimensions by Microscopy and Image Analysis

Particle-Counting Fractal Dimension (D_f)

Assuming a statistically constant microstructural element (or particle) size, the relationship between radius and mass can be altered (eqn. 1.8) to determine the fractality of crystal networks from two-dimensional PLM images (Narine & Marangoni, 1999b; Litwinenko et al., 2002; Campos et al., 2002).

In this case, the scaling relationship between the number of discrete particles (N) and the length of the square region of interest containing the particles (L) can be expressed as:

$$N = cL^D \quad (1.8)$$

where c is a constant, and is N the number of distinct particles within a region of interest of length L . It has been demonstrated that the use of a square region of interest (in lieu of a circle) allows for the computation of the mass fractal dimension (Solecka-Cermakova & Vlckova, 1996). Much like equation 1.1, this method involves an iterative measurement of the number of particles as a function of the size of the decreasing regions of interest (ROI). To begin, the number of particles is first counted within the entire image. At 5% increments, the image is cropped thereby making a new, smaller length L and the number of particles in each new image counted. This process is repeated until the length of the image is 35% of the original size (Marangoni, 2002; Rye, 2003).

Caution must be taken to avoid the occurrence of artefacts in this method. Firstly, the effect of the contribution of small boxes is a skewing of the D value to elevated values. This occurs because as the box size gets small, a tailing off occurs on the log-log plot of $N(L)$ versus L . Once the box sizes become very small, the length of the box sizes decreases faster than the areas of the ROIs. This is especially problematic if the features falling on the edges of the ROI are large, since they will be greatly included and excluded in their respective counts. For this reason, performing the counts on ROIs representing 100% to 35% of the original box size is more accurate (Marangoni, 2002; Rye, 2003).

The number of particles counted within each box size of various lengths (L) is then plotted on a log-log scale, and the slope of the line determined by linear regression. This slope corresponds to D_f . This method for calculating D_f however, was modified from previous procedures in our laboratory in order to eliminate artefacts and improve accuracy (Litwinenko et al., 2002; Marangoni, 2002). Unfortunately, the image analysis macro used is not capable of counting the particles touching only two of the four sides of each ROI (an adjusted count). As a result, including all particles touching all sides of each ROI results in a D value that is lower than expected, and conversely excluding all particles results in a higher than expected D value. To compensate, the average of the D values obtained by including and excluding counts in the 100% to 35% range is taken. This is analogous to counting just those features touching two of the four sides of each ROI, eliminates the skewing effect of small boxes, and results in an accurate D_f value (Litwinenko et al., 2002; Marangoni, 2002). The particle-counting method will be discussed in more detail in sections 2.1.3. and 3.2.11.

Box-Counting Fractal Dimension (D_b)

Another method used to examine microstructure is the box counting or “grid dimension” analysis. This dimension is referred to as a grid dimension because for mathematical convenience the boxes are usually part of a grid that is laid over the image (Barnsley, 1988; Viscek, 1992; Rothchild, 1998; Daoud & van Damme 1999; TruSoft, 2000). The box dimension is defined as the exponent D_b in the relationship:

$$N(L) \sim \frac{1}{L^{D_b}} \quad (1.9)$$

where $N(L)$ is the number of boxes of linear size L necessary to cover a data set of points distributed in a two-dimensional plane. If the network is indeed fractal, plotting the logarithm of $N(L)$ versus the logarithm of L results in a linear plot with a negative slope equal to $-D_b$ (Barnsley, 1988; Viscek, 1992; Rothchild, 1998; Daoud & van Damme 1999; TruSoft, 2000). This analysis appears to be sensitive primarily to the degree of fill of the solids in a crystal network. It can be expected that a network that is more empty (many large void spaces) will result in a lower proportion of full boxes counted, and thus will result in a lower fractal dimension and vice versa (Marangoni & Awad, personal communication, 2003). This type of analysis has been used extensively to examine the fractal dimensions of food protein gels (Hagiwara et al., 1997; Marangoni et al., 2000) and fat crystal networks (Campos et al., 2002; Marangoni, 2002; Marangoni & McGauley, 2003; Rye 2003) and is empirically useful despite the fact the growth of the network is limited to 2-dimensions (Marangoni, 2002). The effects of confined growth on the fractal dimension is therefore one of the aims of the research in this study.

It is important to note that in some cases the fractal dimensions estimated by different methods are not the same. This is well exemplified by the recent studies by Martinez-Lopez et al and by Quevedo et al who found different values of D by number different image analysis techniques (Martinez-Lopez et al., 2001; Quevedo et al., 2002). Values of the fractal dimensions for fat crystal networks determined by the particle-counting and box-counting methods are not equivalent even though both methods are sensitive to the degree of order and amount of mass in the network (Marangoni & Awad, personal communication, 2003). It is therefore necessary that one be aware of the particular method used and its

interpretation; this is mandatory for comparisons of dimensions of different data sets (Russ, 1994; TruSoft, 2000).

1.3.2. Traditional 2-Dimensional Polarized Light Microscopy of Fats

Polarized Light Microscopy (PLM)

The polarized light microscope has been the most widely used form of microscopy in the study of fat crystal networks. PLM involves the manipulation of light in such a way that it allows us to observe crystal structures. In general, light travels from a point source in a propagational or “ray” direction, while having different vibrational directions perpendicular to the wave-front (McCrone et al., 1978; Robinson & Bradbury, 1992). When passed through a sheet of polarizing film, it becomes plane polarized since all of the lights vibrational directions are reduced to a single plane (McCrone et al., 1978; Robinson & Bradbury, 1992). Figure 1.3 demonstrates this concept.

When polarized light interacts with the specimen by double-refraction, the term “birefringence” is used. In the case of observing fat crystal networks, cross-polarization is used, which means that a second polarizer (called the analyzer) is positioned above the sample. When cross-polarization is used, anisotropic crystal structures that doubly-refract are observed as various shades of grey, depending upon their density and orientation. On the other hand, the liquid portion of the network is isotropic, does not refract polarized light, and appears stark black (McCrone et al., 1978; Robinson & Bradbury, 1992). These criteria form the basic means of observing the complex particles and aggregated flocs of particles that make up the fat crystal network, and will be discussed in more detail below.

Figure 1.3: Diagram demonstrating the plane polarization of light. Adapted from McCrone et al. (McCrone et al., 1978).

What is a “particle”?

The majority of quantitative microstructural analyses employed in our laboratory involve the characterization of features that make up the crystal network. Put simply, the thresholding of polarized light micrographs results in the isolation of network components that are of the brighter pixel intensities. This means that the network features that impart ample birefringence are those features that appear as light “flecks”, which are retained upon thresholding. However, because the network can consist of various sizes of crystals and clusters of agglomerated crystals, the concept of individual particle isolation is somewhat confounded. Networks that are more granular in appearance tend to be more easily thresholded and their discrete particles retained. Networks that consist of large clusters of particles become more difficult to threshold for individual crystallites since some merging of particles (both in reality and due to the thresholding process) can occur. This loose definition of a particle leads to criticism worthy of addressing.

Furthermore, not all crystal mass in the network appears as birefringent features that are subsequently retained upon thresholding. This has been demonstrated through the comparison of network features under polarized light and phase contrast (see DVD in Appendix 2 → “Miscellaneous” image gallery, and “Polarized Light-Phase Contrast” movie gallery). In examining these different views of the fat crystal network, it becomes evident that not all mass is represented by PLM imaging. Nonetheless, those birefringent features that are retained following thresholding represent a subset of sampled features that have been shown to be representative of the mass present during early network formation, which dictates the final network properties. The number and distribution of sub-sampled features that are observed in the early stages of crystallization have a profound influence on the final structure, number, and distribution of features as the network continues to reach an equilibrium state (Marangoni & Campos, personal communication, 2003). This indicates that although the appearance of the crystal network may change significantly as it develops, the number of flecks determined by image analysis remains relatively constant, and hence the counting of birefringent features in a fully set network corresponds well to the fundamental particle distribution established in the early stages of crystallization (preceding the occurrence of agglomeration and merging). Recent work by Ph.D. candidate Rodrigo Campos addresses this issue, and will be discussed briefly here.

Figure 1.4 displays the number of particles as a function of time in an experiment studying the effects of shear on cocoa butter microstructure. Corresponding micrographs are presented in Figure 1.5. Note that the number of particles present following storage for extended periods of time remains relatively constant despite changes in the network

appearance, and also that the relative difference in the counts under shear and statically remains nearly unchanged in time.

Operating with this flexible definition of a particle is hence justified, and allows us to perform further analyses including particle size, and fractal dimension determinations with greater confidence.

Figure 1.4: The number of particles (N) as a function of time (h) in a developing cocoa butter crystal network following cooling at 5°C and storage at 24°C. One sample was cooled in static conditions (St), while the other was subjected to shearing (Dy). Data courtesy of Rodrigo Campos.

Thin film crystallization studies

Polarized light microscopy of thin films has been successfully employed to assess the effects of processing conditions on microstructure. Extensive and numerous studies have demonstrated effects on microstructure due to altered rates of cooling (Herrera et al., 1999; Herrera & Hartel, 2000; Wright et al., 2001; Litwinenko et al., 2002; Rye, 2003), the degree of supercooling (de Man, 1964; Foley & Brady, 1984; Wright & Marangoni, 2000; Litwinenko et al., 2002; Marangoni & McGauley, 2003), the presence of shear (Rye, 2003)

and storage time (Rye, 2003). Altering crystallization conditions results in differences in the properties of the network on the microstructural level, which can include changes in the size, morphology, number, and degree of order (Herrera et al., 1999; Herrera & Hartel, 2000; Litwinenko et al., 2002; Rye, 2003). These changes on the microscopic level have been shown to result in changes in the interactions between the particles that make up the network, which in turn affect macroscopic mechanical properties (Litwinenko et al., 2002; Marangoni, 2002; Rye, 2003).

Of particular interest is the fractal scaling behaviour demonstrated by fat crystal networks. 2-Dimensional fractal analyses on images of thin film networks have been extensively studied, and suggest that the relative degree of order and fill in the networks can be quantified, and used to make predictions pertaining to mechanisms of network formation and macroscopic rheological properties (Litwinenko et al., 2002; Marangoni, 2002; Rye, 2003). These analyses have been compared to 3-dimensional fractal dimensions determined by rheological methods with intermediate success (Narine & Marangoni, 1999a; Litwinenko et al., 2002; Marangoni, 2002). This has lead to many questions related to the fundamental differences between 2D and 3D crystallization conditions and their resulting fractal dimensions. Imaging and fractal analysis of fat crystal networks in 3D would therefore be of high value.

Figure 1.5: Polarized light micrographs of cocoa butter cooled at 5°C/min to 24°C statically followed by crystallization and storage at 24°C following A) 1h, B) 3h, C) 6h, D) 12h, E) 24h, and F) 120h. Also shown are image cooled in the presence of shear under the same cooling conditions and following storage for G) 1h, H) 3h, I) 6h, J) 12h, K) 24h, and L) 120h. Images courtesy of Rodrigo Campos.

1.3.3. 3-Dimensional Microscopy of Fats – Previous Efforts

Although polarized light microscopy of thin samples of fats has been of great value in the examination of microstructure, little information about the actual internal 3-dimensional structure of fat crystal networks can be attained by this method. The following discussion briefly highlights some of the significant efforts made to acquire 3D images of fat crystal networks.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy uses a condensed stream of monochromatic electrons which scan the specimen, interact with the sample, and various backscattering electrons are detected and displayed on a cathode ray tube to form an image (Murphy & Roomans, 1984). Of the efforts to image 3D fat crystal networks by electron microscopy the most significant were pursued by Heertje et al. at Unilever Research (Heertje et al., 1987; Heertje et al., 1988). Efforts previous to Heertje et al. were inefficient because thin sections suitable for imaging required freeze fracturing. In preparing samples for fracturing, the freezing process results in the crystallization of the liquid phase of the network which confounds the ability to observe the original solid network structure prior to freezing (Manning & Dimick, 1985). In 1987, Heertje et al. developed a novel sample preparation technique that aimed to remove the liquid phase from the semi-solid 3D fat crystal network while leaving the solid matrix intact (Heertje et al., 1987; de Man & Beers, 1987). Sample preparation required the use of a specially crafted sample holder through which system specific de-oiling of the liquid phase was preformed using mixtures of butanol and methanol at various temperatures and for various treatment times (Heertje et al., 1987). Upon subsequent freezing and fracturing, the

remaining solid matrix was then observed by scanning electron microscopy (SEM). While images obtained by this method successfully demonstrated 3D fat network features, much criticism resulted because in many cases partial stripping of the solid matrix (30-50%) coupled with partial retention of the liquid phase (10-20%) may have resulted in image artefacts (Heertje et al., 1987). Despite this problem, the work of Heertje et al. was successful in demonstrating the general 3D structure of a variety of fat-based food products such as margarines, butter, shortenings, and other fat spreads consisting of oil in water emulsions (Heertje et al., 1987; Heertje et al., 1988; Heertje, 1993). Further efforts by Heertje and Leunis involved removal of the liquid phase using detergents on the surface of bulk crystallized fats, followed by carbon fixation and transmission electron microscopy (TEM) (Heertje & Leunis, 1997). Other significant efforts to image bulk fats by scanning electron microscopy were carried out by Rousseau et al. and involved solvent extraction of solid crystals as well as freeze-fracturing techniques (Rousseau et al., 1996b). This work was successful in demonstrating the presence of both amorphous and defined crystalline regions in tempered milk fat, however, in many cases the crystal structure is not well-defined and the network appears sponge-like in nature (Rousseau & Marangoni, 1999). While these techniques were successful in observing differences in crystal structure due to different processing conditions and composition, their limitations and potential for artefacts resulted in little further use of these techniques.

Fluorescence Confocal Scanning Laser Microscopy (CSLM)

Unlike electron microscopy, which requires complex sample preparations, CLSM allows for 3-dimensional observation of a systems interior without disturbing the internal

structure (Brakenhoff et al., 1988; Brooker, 1991). The principle of confocal microscopy is as follows: light emitted from a source is focused on an imaging pinhole and is reflected by a dichroic mirror on a single point within the specimen. Mirrors on scanning devices then focus the beam in X and Y directions within the sample. Excitation light within the specimen is emitted, passes through the dichroic mirror and is focused on the detector pinhole to form an image of the plane in focus (Bacallo & Garkinkel, 1994). Focusing at various depths can be facilitated by moving the sample or the sample stage in the z-direction, and hence multiple 2D scans can be used to recreate 3D volume renderings (Stevens 1994; Thomas & White, 1998; Verbeek & Boon, 2002)

The use of confocal fluorescence microscopy has been successful as a means of observing fat globules in cheese (Ding & Gunasekaran, 1998) as well as in oil in water emulsions (Van Dalen, 2002). In bulk fat systems, however, attempts to image thick sections ($>50\mu\text{m}$) are met with limited success due to limitations in the ability to image at greater depths. This occurs when emitted light at deep focussing planes is absorbed and scattered and is insufficiently transmitted back through the sample to the detector (Balberg et al., 2000). In addition, the level of structural detail attained by CLSM of fat networks is inherently low, and finer detail within clusters is often indiscernible. Also, speckling can occur due to the presence of residual fluorescent dyes, even after filtering. Due to these limitations, imaging bulk fat systems by confocal microscopy has not been extensively pursued. Figure 1.6 depicts selected slices from a fluorescence confocal z-series that demonstrate these points.

Figure 1.6: Confocal images of anhydrous milk at depths of A) 0 μ m (the top of the sample), B) 20 μ m, and C) 40 μ m. Images by Alejandro Marangoni used with permission.

Recent work by Balberg et al., who have developed a method to extend focus depth in scattering media by use of volume holographic filters, may be of value in the future (Balberg, et al., 2000).

Atomic Force Microscopy (AFM)

Atomic force microscopy is a relatively new technique that can be used to acquire 3-dimensional topographical images. The basic principle of AFM is as follows: a cantilevered tip scans the surface of a sample, and through various measurements of tip deflection and oscillation images can be acquired on the basis of height, amplitude, and phase (Wiesendanger, 1998).

AFM was recently used to study the effects of cocoa butter bloom on the microstructure of the surface of chocolate (Rousseau, 2003). However, the use of AFM to image semi-solid fat crystal networks is difficult due to the presence of liquid oil, which tends to coat the tip of the probe and interfere with signal measurement. Since this method measures surfaces, information pertaining to the interior structures is limited. For this reason,

AFM has not been extensively pursued as a means of imaging bulk fats although some successes in imaging fat crystal networks have been attained, as demonstrated for cocoa butter and for the high-melting fraction of milk fat (HMF) below (Figures 1.7 and 1.8).

All of the aforementioned 3D imaging techniques are valuable in their own respects, but fail to provide sufficient information about the 3D structures in bulk fats systems. The desire to image the interior structures of bulk fat systems in 3-dimensions was the driving force of this thesis study, and the technique developed was based on the concept of transmitted light optical z-series imaging and deconvolution.

Figure 1.7: Atomic force micrographs of cocoa butter at multiple magnifications. The width of each image corresponds to A) 2 μ m, B) 10 μ m, and C) 50 μ m. Images on the left correspond to the height signal, and those on the right correspond to the phase signal. Images with permission by Alejandro Marangoni (Marangoni, 2002).

Figure 1.8: Atomic force micrographs (a 50/50 overlay of the amplitude and phase signals) of HMF at multiple magnifications. The width of each image is corresponds to A) 2 μ m, B) 10 μ m, and C) 25 μ m, and D) 50 μ m.

1.3.4 Imaging and Deconvolution

The Deconvolution Principle

The term deconvolution encompasses a wide range of filtering operations that aim to recover the essential information from a signal degraded by blur and noise (Bones et al., 1993; Kundar & Hatzinakos, 1996) and is not exclusive to image restoration. In fact, deconvolution principles have been extensively studied and implemented in a wide variety of signal processing applications including seismology, acoustics, wireless communications, and non-invasive medical signal analysis (Lanteri et al., 1995; Kunder & Hatzinakos 1996; Lee et al., 1996; Amari & Cichocki 1998; Yu et al., 1999; Protasov et al., 1999; Inouye & Tanebe, 2000; Douglas & Kung, 2000). The commonalities in all of these applications are the desires to undo the convolution (or blurring) observed in a measured signal, and reconstruct the signal close to its original state. The following discussions will focus only on the use of deconvolution in imaging applications.

Applications of Deconvolution in 2-Dimensional Imaging

In most 2D imaging applications, the observed image $g(x,y)$ is a function of the true image $f(x,y)$ and a shift-invariant blur $h(x,y)$, which is also known as the point spread function (PSF):

$$g(x, y) = f(x, y) * h(x, y) + n(x, y) \quad (1.10)$$

where $*$ is the convolution operator and $n(x,y)$ represents all contributions due to additive noise (Kundar & Hatzinakos, 1996). The aim of deconvolution is to remove blur and perform a restoration that best represents the true image $f(x,y)$ having only measured the degraded image $g(x,y)$ and having limited knowledge of the PSF $h(x,y)$ and noise contributions $n(x,y)$ (Law and Lane 1996; Leung & Lane 2000). The model for blur that has evolved in theoretical optics is based on the concept of the PSF. The point spread function is based on an infinitely small point source of light originating in the specimen. Imaging systems collect just a fraction of the light emitted by this point, and therefore cannot focus the light into a perfectly sharp image of the point (Wallace et al., 2001). Instead, the point appears widened and spreads into an XY diffraction pattern. Thus, the point spread function is formally defined as the diffraction pattern generated by an ideal point source of light.

Figure 1.9 depicts a flow chart for the concept of image formation and deconvolution.

Figure 1.9: Flow chart depicting the concept of 2-dimensional image formation and deconvolution principles.

A wide variety of image deconvolution algorithms have been developed and applied, some of which are highlighted below.

Classical 2-Dimensional Deconvolution Algorithms

Inverse filters were the first deconvolution algorithms to be employed in imaging applications, and are commonly referred to as “Wiener Deconvolution”, “Regularized Least Squares”, “Linear Least Squares”, and “Tikhonov-Miller Regularization” (Wallace et al., 2001). In these instances, the PSF is known explicitly prior to deconvolution (Kundur & Hatzinakos, 1996), which allows for accurate knowledge of the convolution process. The algorithm works by taking the Fourier transform of the image and dividing it by the Fourier transform of the explicit PSF, and since division in Fourier space is equivalent to deconvolution in real space, this represents the simplest means of reversing the convolution operator (Javidi, 1990; Wallace et al., 2001). Inverse filtering methods have been used extensively for image deconvolution, with a popular example being images acquired by the Hubble Space Telescope and other astronomical speckle imaging systems (Bones et al., 1993; Kundur & Hatzinakos, 1996). Other common applications include image deconvolution by remote sensing, various medical systems, imaging of the Earth’s underlying surface, and standard photography (Kundur & Hatzinakos, 1996; Christou et al., 1998; Protasov et al., 1999; Goudail et al., 1999). Inverse filters have also been applied to 2D slices of 3D data sets, which will be described in more detail to follow, to improve spatial discrimination in 3D fluorescence imaging (Verveer et al., 2001).

2-Dimensional Deblurring Algorithms

Deblurring algorithms include techniques referred to as “nearest neighbour”, “no-neighbor”, and “unsharp masking” and are another class of 2D deconvolutions. For 2D images, these methods operate under the principle that out-of-focus blur tends to be “flatter” and is composed of lower spatial frequencies than in-focus light. Removal of lower spatial frequencies and boosting of higher spatial frequencies constitutes blur removal (McNally et al., 1999; Wallace et al., 2001). No-neighbour and unsharp-masking are commonly used as artificial focussing operations in image processing software for digital imaging and can be used to enhance poorly focussed 2D photographs, for example (Yli-Harja et al., 2001). Nearest neighbours techniques use some but not all of the 3D information in the 3D data sets to perform plane by plane deconvolution by using information from only adjacent planes to subtract estimated blur (Poon et al., 1992; McNally et al., 1999; Wallace et al., 2001). These algorithms are useful when quick deblurring is desired or when computation power is not available but are often ridden with artefacts due to relative changes in pixel intensities, additive noise from neighbouring planes, and because some features may have PSFs overlapping in the z-direction (Wallace et al., 2001).

2-Dimensional Blind Deconvolution Algorithms

In many cases, knowledge of the PSF is limited, unknown, or difficult and even impossible to measure. Examples of 2D images where this is the case include aerial and astronomical imaging where fluctuations in the PSF due to the atmosphere are common (Kundar & Hatzinakos, 1996). Satellite and ground based telescopes also inherently result in PSFs that are difficult to quantify (Dayton & Sandven, 1995; Bloemhof et al., 2000; Leung &

Lane, 2000). In addition, some imaging scenarios also render measurement of the PSF impossible, such as medical video-conferencing and x-ray imaging (where high intensity beams required for PSF measurement would be hazardous to patients health) (Kundar & Hatzinakos, 1996). In these cases, the use of blind deconvolution algorithms is beneficial because they do not require knowledge of the PSF since the algorithm facilitates both blur identification and image restoration concurrently (Kundar & Hatzinakos, 1996). Blind deconvolution is therefore, an inverse problem of obtaining the best estimate of the true image $f(x,y)$ while concurrently estimating the degradation model or PSF $g(x,y)$ and noise contributions $n(x,y)$ (Dayton et al., 1995; Kundar & Hatzinakos, 1996; Takahashi et al., 1996; Christou et al., 1998; Law & Lane, 1996; Leung & Lane 2000; Yap et al., 2003). Several variations of algorithms and approaches are used for blind deconvolution including: zero sheet separation, *a priori* blur identification, nonparametric deterministic constraints, autoregressive moving average, and high order statistic algorithms (Kundar & Hatzinakos, 1996). Each operates slightly differently, but their functions are similar in that they use the image data to compute the blur function and restore the image.

Applications of Deconvolution to 3-Dimensional Imaging

In the early 1990's, and with great advances in computing power, deconvolution of 3D data sets became more feasible and is now commonly used to remove out-of-focus blur in 3D confocal and fluorescence microscopies (Stevens, 1994; Richardson, 1997). These methods, also referred to as computational optical sectioning microscopy (COSM), involve optical sectioning of 3D volumes by taking a series of 2D (XY) slices at multiple depths

within the sample (the Z axis). A representative diagram of this process is depicted in Figure 1.10.

Figure 1.10: Schematic diagram representing the acquisition of 3D data sets, also called stacks. XY image planes (also called slices) are acquired at known z-increments by use of a motorized microscope stage and contain both in and out of focus features.

Since each optical plane contains both focused features and out-of-focus blur from features above and below each plane, the aim of 3D deconvolution is to either remove this blur, or in more complex algorithms to reassign out-of-focus pixels to their original position within the specimen (Krishnamurthi et al., 1992; Bhattacharyya et al., 1996; Wang et al., 1998; Markham & Conchello, 1998; McNally et al., 1999; Holmes & O'Connor, 2000; Hiesinger et al., 2001; Wallace et al., 2001).

In 3D imaging applications, the observed image $g(x,y,z)$ is a function of the true image $f(x,y,z)$, the 3-dimensional point spread function $h(x,y,z)$ and contributions by noise $n(x,y,z)$ (Markham & Conchello, 1998; Holmes & O'Connor, 2000):

$$g(x, y, z) = f(x, y, z) * h(x, y, z) + n(x, y, z) \quad (1.11)$$

In the case of 3D deconvolution, the PSF takes on a more complicated form, and role. When describing the point spread function in three dimensions, it is useful to apply an x, y, z coordinate system, where x and y are parallel to the focal plane of the specimen and z is parallel to the optical axis of the microscope. The PSF appears as a set of concentric rings in the x-y plane, and resembles an hourglass in the x-z and y-z planes (McNally et al., 1999; Holmes & O'Conner, 2000; Wallace et al., 2001). A representative diagram of the PSF in 3D imaging is depicted in Figure 1.11. Almost all 3D deconvolution algorithms (the exception being blind deconvolution) require knowledge of the PSF. As will be discussed for specific algorithms to follow, the PSF can either be measured experimentally or theorized on the basis of the imaging optics used (McNally et al., 1999; Wallace et al., 2000). To determine the PSF for an optics system experimentally, a sub-resolution fluorescent microsphere (that emulates a point light source) is imaged by taking optical sections starting

above the point source and ending below the point source (McNally et al., 1999).

Figure 1.11: Schematic diagram illustrating the concept of the 3-dimensional point spread function (PSF). Adapted from Holmes and O'Connor (Holmes and O'Connor, 2000).

In addition, the imaging conditions used during PSF determination should best approximate the conditions that will be used to image the actual specimen. To determine the PSF theoretically, software can be used. The information usually required for the computation of a theoretical PSF is as follows: objective lens numerical aperture and working distance, the wavelength of the emitted light, the XYZ image dimensions, the pixel size, the z-spacing, the refractive index of the immersion medium, and the coverslip thickness (McNally et al., 1999).

Aside from the differences in the PSF, the overall process of 3D deconvolution is no different than that of 2D deconvolution depicted in Figure 1.9, except images in 3D are also a function of depth (z).

2-Dimensional Deblurring Algorithms for 3D Data Stacks

Deblurring algorithms techniques referred to as “multiple neighbour” (which was one of the methods used in this thesis study), are essentially 2D deconvolutions that are

performed on 2D slices of 3D stacks of images (Wallace et al., 2001). Multiple nearest neighbours techniques use some but not all of the 3D information in the data to perform plane by plane deconvolution by using information from only a select number of planes above and below each plane to subtract estimated blur (McNally et al., 1999; Wallace et al., 2001). Most commercial deconvolution software, such as the one used in this thesis study, employ a theoretical PSF.

Classical Constrained Iterative Methods: Jansson-Van Cittert, Nonlinear Least Squares

To reduce artefacts that can arise by linear deconvolution methods such as inverse filters, and nearest neighbours algorithms, constraints are imposed that ensure image intensities remain positive (Krishnamurthi et al., 1992; McNally et al., 1999; Wallace et al., 2001). These complex algorithms operate iteratively on the entire 3D data set by first convolving with the input PSF and acquiring a “blurred estimate” which is then compared to the raw images (Wallace et al., 2001). An error criterion is computed via this comparison, which is then used to alter the estimate in such a way that error is reduced. A new iteration is performed, the new estimate convolved, a new error criterion calculated, and so on until the solution is reached at convergence by minimizing the error criterion (Wallace et al., 2001). As a result, the time required to deconvolve is sufficiently greater, however the advantages of artefact reduction may improve results.

Statistical Image Restoration: Maximum Likelihood Estimation (MLE)

The most common type of statistical deconvolution employs a maximum likelihood estimation (MLE) algorithm, which in addition to imposing positivity constraints, also

incorporates information about the statistics of noise (McNally et al., 1999). As a result, these algorithms are more computationally intensive than the classical algorithms, but are better suited to images that are perturbed by appreciable levels of noise (Wallace et al., 2001). Since these methods are also iterative in nature, and more complex, some data sets may require several hours to deconvolve (McNally et al., 1999). These algorithms also require the knowledge of either an empirical or theoretical PSF for the system.

Blind Deconvolution: Modified MLE

As was mentioned in the discussion on 2D blind deconvolution, there are many instances where knowledge of the PSF of the system is either difficult or impossible to acquire. The same applies to 3D microscopy. The main advantage to blind deconvolution is that it obviates the need to have knowledge of the PSF, since it simultaneously computes the blur function and restores the image by using all information in the 3D data set (Krishnamurthi et al., 1992; Bhattacharyya et al., 1996; McNally et al., 1999; Wallace et al., 2001). Blind deconvolution is essentially a modified version of the MLE algorithm, with PSF estimation added (McNally et al., 1999; Wallace et al., 2001). The algorithm starts with a theoretical PSF, but through iteration it adjusts the PSF according to the specific data being deconvolved, handles noise, and to a certain extent can also correct for spherical aberration (Wallace et al., 2001; AutoQuant, 2003). The use of blind deconvolution is therefore an attractive option in many cases, and has been used extensively in various applications using both 3D reflected fluorescence microscopy and confocal (Krishnamurthi et al., 1992; Markham & Conchello, 1998; McNally et al., 1999; Heisinger et al., 2001; Wallace et al., 2001). Some commercially available blind deconvolution software packages now even

include algorithms that correct noise and artefacts inherent to CCD cameras prior to deconvolving (Wang et al., 1998).

A Note on Deconvolution and the Potential for Vendor-Driven Biases

The choice of empirical or theoretical PSF use is often a point of contention amongst users and developers on deconvolution algorithms. While some believe that the empirically measured PSF is easy to acquire and gives superior results compared to the use of a theorized PSF, others believe that empirical measurement of the PSF is rigorous and if done incorrectly can impair the images more than improve them. Others believe that for various imaging scenarios, the empirical point spread function may differ significantly from the PSF that occurs when imaging the actual specimen and that in some cases the use of a theoretical PSF may be preferred since it is noise-free.

Another point of contention amongst deconvolution users and software developers is the use of a blind deconvolution algorithm in lieu of measuring and using an empirical PSF and non-blind algorithm. While some believe that an accurately obtained empirical PSF is best, others would argue that blind deconvolution is a better alternative since any inaccuracies in the empirical PSF would be expressed in the deconvolved images. Since deconvolution users are also involved in the development and sale of expensive deconvolution software the potential for bias arises, therefore it is important that this be considered when looking for a deconvolution solution. In many instances the right choice of algorithm or software is dependant on the intended use. In some cases, several algorithms may perform equally well. Ideally, a number of algorithms should be explored. For this reason, many vendors of deconvolution software offer temporary demo software and/or

encourage the submission of experimental data for demonstrative purposes prior to purchase. These discussions can be found by searching the archives of the University of Buffalo Confocal Listserv (University of Buffalo, 2003).

Adaptation of Deconvolution Techniques to 3-Dimensional Transmitted Brightfield (TLB) Microscopy

The adaptation of a blind deconvolution algorithm for use in 3D transmitted light brightfield microscopy was first proposed by Holmes et al. in 1995, and was especially attractive since until then there wasn't a robust means to image in 3-dimensions by transmitted light (Holmes et al., 1995). Since TLB is also one of the most widely used imaging modalities in a number of scientific disciplines, and since blind deconvolution can be used pervasively, this development has the potential for great impact (Holmes & O'Connor, 2000).

The deconvolution algorithm proposed for TLB is based on the MLE blind approach discussed previously, with a number of added components. These include an estimate of the 3D optical density distribution (also positively constrained), and a "guardband" which constrains the bulk of the energy within the PSF to lie within an hour-glass shaped 3D region (which serves to eliminate artefacts caused by circular convolution) (Holmes & O'Connor, 2000). During the development of this algorithm, an image montage and an acceleration scheme were also devised as a means of reducing the number of required iterations to convergence (Holmes & O'Connor, 2000). Another important aspect of this recent development was that it demonstrated the possibility for 3D imaging and deconvolution by a modality that has a partially coherent optics model (Holmes & O'Connor, 2000).

Preliminary applications of the algorithm on stained pyramidal neurones resulted in identifying dendrite features by TLB that were not easily observed by 3D fluorescence or confocal microscopies (Holmes & O'Conner, 2000). This recent deconvolution development, in addition to preliminary discussions with Dr. Tim Holmes, served as a major source of inspiration and confidence that the adaptation of optical sectioning and deconvolution to transmitted polarized light microscopy should be pursued (Holmes, personal communication, 2002).

2. METHODS AND MATERIALS

This section addresses the methodologies employed in this study. This includes the preparation, imaging, image processing, and image analysis of 2-dimensional and 3-dimensional systems. The model fat system used in all studies was the high-melting fraction of milk fat (HMF) diluted in triolein at a 30:70 (w/w) ratio.

2.1. Conventional 2-Dimensional (Thin Film) Methods & Materials

2.1.1. Conventional 2D (thin film) Slide Preparation

The following method is the standard procedure for preparing samples for imaging, and has been employed in our laboratory for many years and for many fat systems for various crystallization experiments (Narine & Marangoni 1999b,c; Wright et al., 2000; Wright & Marangoni 2002; Campos et al., 2002; Litwinenko et al., 2002; Marangoni & McGauley, 2003, Rye, 2003).

A small, representative sample of the fat was first melted and held at 80°C in an oven for a period of 30 minutes, to ensure that all crystal memory was erased. Using a preheated, thin, glass disposable micropipette and plunger (Drummond Scientific Company, Broomall, PA, USA), molten fat was drawn from the mixed, molten sample. Working in the oven, the plunger was held perpendicular to a preheated 25x75x1mm glass slide (Fisher Scientific, Pittsburgh, PA, USA) and the plunger was slowly depressed until a small droplet (approximately 10 μ L) of molten fat fell onto the slide. A 22x22mm preheated glass cover

slip (Fisher Scientific, Pittsburgh, PA, USA) was then carefully placed over the sample to produce a film of uniform thickness. If this is performed carefully, no excess liquid fat should be expelled from under the cover slip, thereby ensuring a consistent thickness within the sample, and in repeated slide preparations.

The slide was then quickly transferred to a thermostatically controlled microscope stage (Model LTS 350, Linkam, UK) which was preheated to 80°C (Figure 2.1).

Figure 2.1: The Linkam LTS 350 microscope heating/cooling stage. The temperature of the sample is maintained by the use of a heating coil and liquid nitrogen, which are controlled by an external programmer and pump system (not shown).

Each sample was immediately viewed under the microscope to confirm that it remained in a completely molten state (no birefringence observed) during transfer from the

oven to the imaging system. The sample was then held at 80°C for 10 minutes to ensure a consistent initial temperature, and cooled at a controlled rate of 10°C/min (mediated by the temperature-controlled stages external pump and programmer, which uses liquid nitrogen as a cooling medium) to 28°C and held isothermally for 15 minutes prior to imaging. This process was repeated for three slides.

2.1.2. Conventional 2D (thin film) Imaging

Imaging of the thin films was performed on a microscope using polarized light (Leica Microsystems: DM-RXA2/CTR-MIC - Richmond Hill, ON, Canada). The polarizer was set to 90° (full extinction) relative to the analyzer (crossed polarization). The light source was set at 10.5 volts and the aperture was wide open. The objective lens used had a numerical aperture of 0.55 and magnification of 50X. The aperture was closed down until it was visible in the field of view, centered, and then opened to just beyond the field of view in order to ensure even and efficient illumination. A green filter (Hoya: G(X1) - Japan) was used to provide monochromatic light and enhance contrast (McCrone et al., 1978).

A cooled CCD camera (Q-Imaging: Retiga 1300 - Burnaby, BC, Canada) was used to view the magnified sample on a CRT monitor (LaCie: Electron 19 blue III - Hillsboro, OR, USA) on a personal computer (Apple: Macintosh G4 – Cupertino, CA, USA). Exposure was set to ensure a proper distribution of greys (i.e. the features were visible, but no saturation of pixels occurred). This was confirmed by viewing the live camera image histogram, which displayed all pixels frequencies within the range of 0-255 greyscales. The lighting conditions and camera exposure were kept constant during imaging to avoid introducing variables.

Focused images were acquired as uncompressed 8-bit (256 greys) greyscale TIFF files, with a spatial resolution of 1280x1024. Three slides were prepared as previously described (section 2.1.1), and three random, representative fields were imaged from each slide.

In various past studies in our laboratory a different microscope (Olympus: BH - Japan), video CCD camera (Sony: XC-75 - Japan), and image capture board (Scion: LG-3 – Frederick, MD, USA) with on board 16-frame averaging were used as our primary imaging system (Wright et al., 2000; Wright & Marangoni 2002; Campos et al., 2002; Litwinenko et al., 2002; Marangoni & McGauley, 2003). This setup resulted in 8-bit greyscale images with a spatial resolution of 640x480 pixels (and hence a smaller field of view acquired in each image, relative to the newer imaging system). It was therefore, in our interest, to compare both qualitatively and quantitatively, results of imaging experiments from the old and new microscope and camera imaging systems. In particular, our interests lay in determining whether or not the change to newer optics and image resolution had any effects on the image analysis parameters D_b and D_f , which are fractal dimensions that describe the order and fill of the crystal networks (to be explained in more detail in section 3.1.2). This comparison will be addressed as a brief note in the results (section 3.2.11).

2.1.3. Conventional 2D (Thin Film) Image Processing & Analysis

Thresholding

Greyscale images of the network must be converted to a binary format prior to image analysis. The discriminatory conversion of greyscale images to binary format, requires a process termed “thresholding”. Images of fat crystal networks are typically obtained as 8-bit

(256 color) images and consist of a histogram of pixel values that span the range of 0 (pure white) and 255 (pure black). Thresholding involves the selection of a particular pixel value within the range of 0 and 255 that will result in a binary image that best isolates the features of the network. This process is subject to much controversy since the selection of the cut-off pixel value can often be subjective. To minimize subjectivity, an auto-thresholding algorithm can be used that often provides accurate and reproducible results¹. This algorithm works by scanning the histogram to find an intensity value where the average moments of the histogram counts about an intensity value are balanced. This means that a threshold value is chosen where the average pixel intensities are equal above and below the threshold (Joss, 2002). The general process of thresholding can be seen in Figure 2.2.

When auto-thresholding fails to result in a binary image that accurately represents the features in a greyscale image, manual, and somewhat subjective thresholding can be performed. Manual thresholding is subject to much criticism because it introduces variability in the user's subjective determination of threshold. In order to reduce this subjectivity as much as possible, the following procedure is performed in Adobe Photoshop (Adobe Systems Inc, CA, USA): the image is opened, inverted, and an exact copy is overlaid on a layer above the original. This top, duplicated layer is then thresholded. By toggling the opacity of this thresholded layer, a scrupulous comparison to the underlying original greyscale image can then be made, and the threshold value adjusted accordingly (if the first chosen value does not accurately represent the features of the original image).

¹ Performed on a Macintosh computer using the public domain NIH Image program, developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>

Figure 2.2: The conversion of an 8-bit (256-greyscale) micrograph to a binary (pure black and white) image suitable for image analysis. A threshold value is automatically or manually chosen to isolate the features that will be measured.

This procedure was followed for all required manual thresholding operations in this study. Once the image is converted to binary and the features isolated, it can be subjected to image analysis. Features are measured, and result in data, which can then be interpreted and related to structure. A flow chart outlining the general process from start to finish is depicted in Figure 2.3 (Russ, 1991). Emphasis is put on acquiring initial images that are of high quality (high distribution of pixel values, no saturation of white features, and evenly distributed lighting) in order to avoid unnecessary image processing to enhance features thereby altering pixel values.

Image Calibration

Brightfield images of a 1000 μm stage micrometer (Fisher Scientific, Pittsburgh, PA, USA) were acquired and used to create an image calibration file prior to performing all analyses. The Image Processing Toolkit (IPTK) processing and analysis filter/plugin package (Reindeer Graphics, Asheville, NC, USA) automates various imaging tasks, and works within the framework of the graphics software Adobe Photoshop (Adobe Systems Inc, San Jose, CA, USA). Using the IPTK's IP-Measure $\rightarrow$ calibrate filter, two points on the image of the micrometer (of known distance apart) were marked and the corresponding known length input. This filter results in a calibration file that saves the $\mu\text{m}/\text{pixel}$ ratio and subsequently allows all further quantitative imaging filters to output calibrated results in units of μm .

IMAGE ANALYSIS OF MICROSTRUCTURE

Figure 2.3: Flowchart depicting the steps involved in obtaining and evaluating the microstructure of materials from digitally acquired images (adapted from Russ, 1991).

Fractal Dimensions

Assuming a statistically constant microstructural element (or particle) size, the relationship between radius and mass (eqn. 2.1) can be used to determine the fractality of crystal networks from two-dimensional PLM images (Narine & Marangoni, 1999a,b).

In this case the scaling relationship between the number of discrete particles (N) and the length of the region of interest containing the particles (L) can be expressed as:

$$N = cL^D \quad (2.1)$$

The first method used was a particle counting algorithm performed using NIH-Image² as follows: c is a constant, the number of distinct particles (N) are first counted within the entire image of known length (L). At 5% increments, the image is cropped thereby making a new, smaller length L and the number of particles in each new image counted. This process is repeated until the length of the image is 35% of the original size. A diagram depicting the overlaying of the boxes of decreasing size can be appreciated in Figure 2.4.

The number of particles counted within each box size of various lengths (L) is the plotted on a log-log scale, and the slope of the line determined by linear regression. This slope corresponds to D_f . This method for calculating D_f was modified from previous procedures in our laboratory in order to eliminate artefacts and improve accuracy. This was accomplished by performing these iterative counts twice on each image – once including all particles touching the edge of each region of interest, and once excluding those that touch the edges. By taking the average of these two counts, a D_f that best represents the spatial

² Analysis performed on a Macintosh computer using the public domain NIH Image program, developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>

distribution of mass is obtained (Campos et al., 2002; Litwinenko et al., 2002; Marangoni, 2002). This improved method is equivalent to well-established methods that involve counting features that touch two sides of the region of interest (Russ, 1999). A brief discussion of the effects of edge and image resolution on the particle-counting fractal dimension will be included in greater detail in the results (section 3.2.11).

Figure 2.4: Schematic diagram showing the iterative decreases in box size (or region of interest – ROI) used for the particle counting method for the determination of the fractal dimension D_f .

The fractal dimension arrived at by this method reveals information on the degree of order in the packing of the microstructural elements within the microstructures. Systems that display a high degree of disorder have characteristically lower fractal dimensions than those of a more ordered network (Viscek, 1992; Rothchild, 1998; Narine & Marangoni, 1999b; Litwinenko et al., 2002). Another method used to examine microstructure is the box counting or “grid dimension” analysis. This dimension is referred to as a grid dimension because for mathematical convenience the boxes are usually part of a grid that is laid over the image (Barnsley, 1988; Viscek, 1992; Rothchild, 1998; Daoud & van Damme 1999; TruSoft, 2000). The box dimension is defined as the exponent D_b in the relationship:

$$N(L) \sim \frac{1}{L^{D_b}} \quad (2.2)$$

where $N(L)$ is the number of boxes of linear size L necessary to cover a data set of points distributed in a two-dimensional plane. If the network is indeed fractal, plotting the logarithm of $N(L)$ versus the logarithm of L results in a linear plot with a negative slope equal to $-D_b$ (Barnsley, 1988; Viscek, 1992; Rothchild, 1998; Daoud & van Damme 1999; TruSoft, 2000). This analysis appears to be sensitive primarily to the degree of fill of the solids in a crystal network. It can be expected that a network that is more empty (many large void spaces) will result in a lower proportion of full boxes counted, and thus will result in a lower fractal dimension and vice versa (Marangoni & Awad, personal communications, 2003). A screen capture depicting the software interface is included in Figure 2.5.

It is important to note that in some cases the fractal dimensions estimated by different methods are not the same. It is therefore necessary that one be aware of the particular method used and its interpretation; this is mandatory for comparisons of dimensions of different data sets (Russ, 1994; TruSoft, 2000).

Figure 2.5: Screen capture depicting the box-counting fractal dimension (D_b) determination in TruSoft's Benoit software application. Box size is iteratively reduced and the number of occupied boxes counted. Results are plotted on log-log axes, and the resulting slope is $-D_b$.

Area Fraction

Area fraction was determined from binary images by using the IPTK's IP-measure→global filter (Reindeer Graphics, North Carolina, USA). This filter works by simply counting the number of black pixels which represent crystal mass. The number of black pixels divided by the total number of pixels in the entire image represents the area fraction, which is an indication of the quantity of crystal mass.

Number of Particles and Microstructural Element Size

The number of particles, N , (as was defined in section 1.3.2) within each 2-dimensional image was computed using NIH-image³. The first step of the particle counting fractal dimension determination involves the counting of discrete features within the full image dimensions. This count is performed twice, once ignoring all features that touch the edge of the region of interest, and a second time including these edge touching features in the count. The number of particles was taken as the average of these two counts, and is equivalent to ignoring the particles that touch two of the four edges of the region of interest. Alternatively, and with greater ease, the number of particles can be computed using the IPTK's IP-features→distribution filter. This filter can be used to count all particles in the image neglecting edge effects (less accurate), or can be used to perform an "adjusted count" which takes into account the number of edge-touching features on the basis of their size distribution (more accurate) (Russ, 1999). Results from the NIH-Image and Photoshop methods are nearly equivalent, but since the data for number of particles were already

³ Analysis performed on a Macintosh computer using the public domain NIH Image program, developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>

produced within the data used to compute the particle-counting fractal dimension, there was no need to repeat counts in Photoshop.

Mean microstructural element size (MES) was determined concurrently with the IPTK's IP-features → distribution → size (based on area) filter, and makes no assumptions regarding geometry. By counting the number of pixels a feature occupies, and referencing the $\mu\text{m}/\text{pixel}$ calibration, it outputs the result as an area in μm^2 .

Mean Nearest Neighbour Distance (NND)

In order to lend support to the belief that fractal dimension analyses reveal information about the degree of order in the network, mean nearest neighbour distances were calculated. Of particular interest is not necessarily the value of the NND (which would be indicative of the relative “compactness” of the network particles), but rather the standard deviation of the NND, which would indicate the variation in distances between particles. If the variation in the calculation of the mean NND is high, it would be indicative of a more disorderly distribution of particles, whereas a lower relative variation would indicate a more orderly distribution.

2.2. Novel 3-Dimensional (Volume) Methods & Materials

2.2.1. 3-Dimensional Slide Preparation

Initial attempts to create a volume suitable for imaging were performed in the following manner: A metal washer of approximately 300 μm in thickness was placed on a

glass slide. The slide and the mental washer were preheated to 80°C and the molten fat sample was poured into the ring-shaped washer. A preheated glass cover slip was then placed on top of the ring and slight pressure was applied to ensure good contact between the cover slip and the washer. Excess molten fat was therefore forced out by this pressure. The sample, still in the melt, was then transferred to the temperature controlled stage apparatus which was at a temperature of 80°C. Preliminary experiments with a cooling rate of 10°C per minute to 28°C followed by isothermal crystallization for fifteen minutes yielded a highly birefringent fat crystal network, capable of being imaged slice by slice all the way through the volume. However, within a very short period of time, the network became excessively birefringent and it became difficult to discern individual crystal agglomerates.

Further attempts to create a volume that was suitable for imaging involved a reduction in the volume thickness. An attempt to source slides with wells of known depth that weren't curved in shape was unsuccessful. Slides with manufactured wells also presented a further difficulty since the depth was variable at any XY position.

A sample preparation that resulted in a suitable volume was performed in the following manner: a standard glass slide formed the base of the sample holder. Two glass cover slips were glued to the glass slide using liquid crazy glue leaving a gap of 8mm between them. Molten fat sample was then placed within this gap and a third preheated glass cover slip was placed over the gap. Access molten fat sample was forced to out by applying slight pressure. In order to be certain of sample thickness, a small scratch was etched onto the surface of the glass slide, and on the underside of the final cover slip. These small scratches could be viewed under the microscope, and the position of the z controlled stage recorded. This established the top and bottom of the sample, and the overall sample thickness was

determined to be $207\mu\text{m}$. This depth is greater than that of a standard glass slide ($170\mu\text{m}$) likely due to small contributions in height added by the thin layer of supporting glue and any remaining liquid fat that may have been present between the fixed bottom cover slips at the third cover slip that bridged them from above. A representative schematic drawing is presented in figure 2.6.

Figure 2.6: Top view and profile view of the apparatus used to create a $207\mu\text{m}$ thick sample suitable for imaging as a stack. Two standard glass slides (light grey) were glued to a standard glass slide (dark grey). Molten fat (black) was poured into the gap between the two glued slides, and a third glass slide (light grey) was placed on top with slight pressure. Note: components are not shown to scale.

When a sample in this apparatus was crystallized in the same manner as the washer apparatus, a birefringent crystal fat network was established and could be imaged for an extended period of time without becoming too birefringent, and without changing appreciably in time.

2.2.2. 3-Dimensional Imaging

Imaging of thick sections was performed using the same imaging apparatus as previously described for thin films (section 2.1.2). The only modification to the imaging technique was a change in the aperture setting, which in the case of thick sections, was opened to a wider extent in order to achieve a reduced focusing depth (McCrone et al., 1978).

Prior to imaging, the sample apparatus was viewed under brightfield conditions in order to establish the z-stage position representing the top and bottom of the actual sample. This was achieved by recording the position of the stage when the finely scratched glass surfaces were in focus.

All imaging was performed within the Openlab software module (Improvision Software and Scientific Imaging Inc, UK). The software includes automations that allow for user input of the following parameters: the stage position at the top and bottom imaging planes, and the number of optical slices desired between these two stage positions. The top and bottom focus planes were set in accordance to the positions of the scratches on the sample holder glass surfaces, as described in section 2.2.1.

The camera and computer interface used were the same as described for the imaging of thin films (section 2.1.2.). Once again, the exposure level was appropriately set to ensure a proper distribution of pixel intensities and the histogram was consulted in order to confirm the appropriateness of the selected exposure time.

The z-series automation (macro) was loaded and started. A total of 150 optical slices were acquired between the top and bottom set-points, and the resulting z-increment was 1.38 micrometers per slice. The sample thickness was a total of 207 μ m. Images were acquired as

8-bit greyscale (256 greys) with an image resolution of 1280x1024 pixels. Each resulting slice was 1.3Mb in size, and the total imaged volume approximately 192Mb.

Upon completion of the z-series acquisition, the entire data set was then saved in Openlab's proprietary "LIFF" format, which saves all image and layer information in one file. By selecting all imaged layers, and accessing the file drop-down menu, the entire stack of 2D slices was output as individual numerically ordered 8-bit TIFF files, which were later analyzed. A 2D projection of the entire stack was prepared, as will be discussed in section 2.2.3. This image was then saved as a separate 8-bit TIFF file.

2.2.3. 3-Dimensional Image Processing

Image Deconvolution – Nearest Neighbours (NN) Algorithm

Image deconvolution was preformed by two different algorithms. The first deconvolution technique used was a component of an image processing application called Openlab 3.1.4, which was developed by Improvision (Improvision Software and Scientific Imaging Inc, UK) and distributed by Quorum Technologies (Quorum Technologies Inc, Guelph, Canada). The deconvolution was performed on the same Macintosh computer system used for the imaging (section 2.1.2). The Openlab deconvolution algorithm employs a "multiple neighbours" technique, for which the background theory was described previously in more detail in section 1.3.4 of the literature review, and briefly reiterated here. In this case, a theoretical point spread function is used to perform the deconvolution (McNally et al., 1999; Wallace et al., 2001).

Following acquisition of the raw 150 slice volume stack, all layers were selected, and the volume deconvolution option in the image drop-down menu was chosen. Figure 2.7 depicts the working interface for performing the deconvolution. In order to apply the algorithm to 3D data sets obtained using a polarized light modality (which to date has not been presented in literature) the theoretical point spread function was input as a fluorescence modality. This assumption will be addressed in more detail in section 4.2.

Figure 2.7: Screen capture depicting the deconvolution dialogue box in the Openlab Volume Deconvolution software application. Input requirements for nearest neighbours (8 in this case) deconvolution include: sample z-spacing; microscope lens magnification, numerical aperture, medium refractive index, and emission wavelength. Camera pixel size and binning values are also required. A magnified preview image, and navigation within the stack assess the effects of the chosen haze removal, gain, and offset values.

Input requirements include a value for the number of nearest neighbours. Increasing the number of nearest neighbours increases the accuracy of the deconvolution but

concurrently increases the time required to perform the deconvolution. Preliminary efforts on images of dimension 1280x1024x150 employing 4 nearest neighbours required approximately 1 hour to deconvolve and resulted in mediocre results (not shown). Increasing the number of nearest neighbours to a value of 8 (which means that each slice is deconvolved by referencing four slices above and four slices below each layer) required approximately 3 hours for deconvolution and yielded significantly better results. This value was therefore deemed appropriate.

Input requirements related to the computation of the theoretical point spread function included the sample z-spacing ($1.38\mu\text{m}$), microscope lens magnification (50X), numerical aperture (0.55), immersion medium refractive index (air = 1), emission wavelength (595nm), camera pixel size (6.64) and binning value (1).

A magnified preview image and slider bar, which allowed for navigation within the stack, gave a real-time assessment of the effects of the chosen haze removal, gain, offset, smoothing, and shadow values. The gain and offset values are analogous to controlling the deconvolved images brightness and contrast values, and were set to values that best represented the refocused structures and avoided pixel saturation (Improvision, 2000). Smoothing reduces the contrast, especially at the edges of features, and was therefore not used. Shadows introduce artificial edge effects, and hence were not used (Improvision, 2000).

By altering the degree of haze removal, a subjective decision as to what value is appropriate must be made. In other words, by toggling the haze removal value, the user has the ability to alter pixel values, thereby including or excluding haze on the basis of a visual

preview. Since the haze removal is not perfect, by toggling the haze removal value to too great an extent, it appeared to remove actual focused features. This is a well-known and characterized problem with several deconvolution techniques (Wallace et al., 2001). In order to address this issue, it was decided that a strategy must be implemented to avoid removing features that should be retained. By navigating into various depth positions within the stack, where focused features were present, and toggling the haze removal value to a maximum for each layer, a global “threshold haze removal value” could be determined. This value was such that it did not result in the removal of any actual focused features within the entire 150 slice stack. As a consequence of meeting this requirement, all unfocussed haze could not be eliminated; however, enough was removed to produce an improvement in focus at each optical imaging plane.

Image Deconvolution – Blind Algorithm

The second deconvolution technique used was an adaptive blind algorithm found within the application called AutoDeblur & AutoVisualize 9.1, which was developed by AutoQuant (AutoQuant Imaging Inc, New York, USA). A 30-day full access trial version was downloaded free of cost from <http://www.aqi.com>. Deconvolution was performed on a Toshiba Satellite 2450 laptop PC with a 2.6GHz processor and 512Mb of RAM. Unfortunately, repeated attempts to deconvolve a 3D data set of size 1280x1024x150 (192Mb) were unsuccessful due to computer failure, even with what was assumed to be ample processing power and memory. To solve this problem, the size of the data set was reduced to 640x512x150 (48Mb) by batch resizing using “constrained aspect ratio” and

“smooth bicubic interpolation” in Photoshop. It must be noted that this step-down in size is not lossless, but rather is analogous to a minor image compression, since groups of pixels are grouped into single pixels by interpolation (Adobe, 2002).

The AutoQuant deconvolution algorithm employs an “adaptive blind” technique, for which the background theory was described previously in more detail in section 1.3.4 of the literature review, and briefly reiterated here. In this case, an empirically determined point spread function, computed from the actual data set is used to perform the deconvolution (AutoQuant, 2003).

The numerically sequenced raw 150 slice volume stack was opened in the AutoQuant software. By default, the software displays the stack as one 2D brightest point merge projection image. To view the stack as slices, the slice viewer icon need be accessed. The deconvolution is performed by accessing the “Deconvolution” drop-down menu and selecting “Start 3D Deconvolution” tab. Figure 2.8 depicts the working interface for performing the deconvolution.

Once again, in order to apply the algorithm to 3D data sets obtained using a polarized light modality the initial estimated point spread function was input assuming a fluorescence optics modality. This included the sample z-spacing ($1.38\mu\text{m}$), microscope lens magnification (50X), numerical aperture (0.55), immersion medium refractive index (air = 1), emission wavelength (595nm). From this initial information, and from the scattering detected from the actual 3D data set, the empirical point spread function is automatically generated (AutoQuant, 2003). The automatic deconvolution settings were used, which incorporates an iteration and save interval of 10, file output as 16-bit TIFF, and low noise

level. This resulted in the best stability, high resolution, and moderate speed. The resulting PSF was saved as a proprietary .DEB file (630Mb). This saved PSF file could potentially be used in future non-blind constrained iterative deconvolutions, if deemed appropriate as a representative theoretical PSF for other specimens. Spherical aberration was assumed to be negligible, and hence was not incorporated into the deconvolution process. For the sake of interest, however, spherical aberration detection was run, and resulted in a value of -0.87 on a scale spanning the range of -15 to 15, indicating little to no influence. Deconvolution required approximately 1.5h for completion, after which the deconvolved 3D data set was then saved as individual 8-bit TIFF files.

Figure 2.8: Screen capture depicting the deconvolution dialogue box in the AutoQuant AutoDeblur software application. Input requirements for blind deconvolution include: image X, Y, and Z-spacings; microscope lens numerical aperture, immersion medium refractive index, and modality; and emission wavelength.

2D Projections of 3D Data Sets

2-Dimensional projections of the entire 150 slices of each stack were prepared in order to examine the effects of projection of 3-dimensional features onto 2D Euclidean space. The technique employed was a “Brightest Point Merge” or “maximum projection”. Each projection was prepared in its respective software bundle. The NN data set was projected in the Openlab software by selecting all 150 layers simultaneously and dragging them onto the “new layer” icon in the layers palette. The software examines each XY coordinate or pixel along the Z direction or “rays”, and displays a new layer which represents the 2D projection of the full stack (NIH-Image, 2003). By projecting the brightest point encountered along each z-oriented ray, the brightest objects are displayed. In the AutoQuant software package, when a stack of images is opened, it by default displays the maximum point projection. To save this projection as a single image, one must simply “save the current view” under the file drop-down menu. In all cases, the output image format was 8-bit TIFF.

Each 2D projection was then analyzed in the same manner as all other 2D images. Images were manually thresholded to isolate discrete features. This required a threshold value of 220 for the NN data set, and a value of 228 for the blind.

3D Renderings and Animations

3-dimensional volume renderings were prepared as a means of visualizing the stack of 2D images, and were not meant to be used for quantification. Qualitative comparisons of the effects of the nearest neighbours and blind deconvolution on the 3D structure could, however, be made.

A 3-dimensional rotating volume rendering was prepared for each of the three data sets (raw, NN, and Blind) using the Autovisualize module in the AutoQuant software bundle (AutoQuant Imaging Inc, New York, USA). Preparing 3D renderings of the full resolution images (1280x1024x150) was not possible because of the intense computing power required (this was attempted, but computer crashing/hanging resulted). Images were resized to 640x512, but the same problems occurred. Images resized to 320x256, were however, of suitable size for the rendering to proceed. Images were batch resized by creating an automation in Adobe Photoshop 7.0 (Adobe Systems Inc, San Jose, CA, USA). Sequentially numbered images were each opened and resized to 320x256 with the “constrain proportions” and “smooth bicubic resampling” options activated. This operation reduced the XY resolution and the size of each image to 82kb (which in turn made the full 150 slice stack a workable 12Mb). This step-down in size is analogous to image compression, since groups of pixels are grouped into single pixels by interpolation (Adobe Systems, 2003). This significant step-down in image size also justifies the use of resulting 3D renderings for qualitative purposes only.

The entire stack of sequentially numbered images was opened in the AutoQuant application, and the appropriate X, Y, and Z aspect ratio was input. The “make movie” option in the AutoVisualize drop-down menu was then selected. The software automatically creates a series of 2D projections of the 3D data at various observation angles. To customize the 3D volume rendering, the options drop-down menu in the 3D viewer window was chosen, and the control panel accessed. The rendering mode chosen was “volume rendering – hardware” which is preferred over the “software” option since it creates smoother rotational transitions (AutoQuant Imaging, 2003). The maximum projection mode was selected since it

resulted in the best visual representation of the structure. Under the “movies” tab in the 3D viewer control panel, a rotation about the Y-axis from the initial XY starting position to 360° was applied. A step angle of 5° and frames/sec value of 40 were chosen to ensure reasonably smooth rotation and movie quality. The display perspective angle was set to 40° and the hardware volume projection quality setting set to highest. A preview window allowed the user to see the resulting movie file and the “create movie” button created the full rotating series as an AVI file (compatible with most media players).

All movies (raw, NN, and blind) were prepared in the same manner, using the same rendering options. A fourth, anaglyphic movie was created from the blind deconvolved data set, and included the addition of stereoscopic effects to further enhance the 3D depth qualities. Within the movie control panel settings/stereo tab, a red/cyan filter was applied at a +/- angle of 5°. This digital image filter adds a shifted red and cyan colour channel to the image features, which when viewed through actual red/cyan filters, creates a perception of added depth (Russ, 1999).

Since 3-Dimensional volume renderings can not be fully appreciated and evaluated in the form of 2D printed images, they are included in digital format on the DVD in the Appendix. To access them, the HTML file entitled “index” should be opened in any web browser on a Macintosh or PC computer capable of viewing AVI file types. By following the “Movies” link, the 3D rendered movie files should be readily located within.

2.2.4. 3-Dimensional Image Analysis

Without the acquisition and use of very expensive 3D analysis software, information about the 3D dimensional fat crystal network can still be obtained. This was attained by two

methods: firstly by performing analysis on each slice as an independent image, and secondly by creating a 2-dimensional representation or “projection” of all the slices that made up the 3D volume.

2D Slices from 3D Data Sets

The 2D images that made up the raw 3D z-series contained too much haze and out of focus features to confidently and accurately segment true features via thresholding. For this reason, analysis of the raw 3D data sets was not performed.

2D images that resulted after deconvolution by both the nearest neighbours and blind algorithms, however, were suitable for thresholding because ample haze was removed to allow for discrete feature isolation. Automatic thresholding (see section 2.1.3) was not an option for converting these data sets to binary images prior to analysis, since the resulting binary images did not adequately or accurately represent the features present. Manual thresholding was performed in the same manner as it was in the binarization of thin section images in section 2.1.3.

For consistency, a constant threshold value was used for all images within each 3D stack. After performing the procedure for determining an accurate thresholding value in a subjective manner (see section 2.1.3) in every 10th slice in each stack, the same threshold was applied to all images in each stack. The NN data set was thresholded at a value of 220 and the blind stack was thresholded at a value of 250. All binary images were analyzed by the same methods described for thin films (section 2.1.3). The XY $\mu\text{m}/\text{pixel}$ ratio was assumed to be independent of position in the Z direction, and the same calibration file used in thin film studies was therefore used for all slices in the 3D volume.

2D Projections

Each set of 2D binary images was then analyzed in the same manner as conventional 2D images of thin sections, including the area fraction, number of discrete particles, mean particle size, and fractal dimensions D_b and D_f (section 2.1.3).

In addition to these analyses, a quantitative fast Fourier transform (FFT) image analysis technique was used to qualitatively demonstrate the degree of haze removal in projections following NN and blind deconvolutions. After selecting a 512x512 pixel region of interest from each image 2D projection (raw, NN, and blind), the IPTK filter IP-FFT → forward FT was applied. This filter converts the spatial domain to the frequency domain, and replaces the original image with the power spectrum (Russ, 1999). The resulting patterns in the power spectrums were then examined for the presence of characteristic banding which is indicative of focus quality and in this case the presence of haze (Russ, 1999).

Pearson Linear Correlation Analysis

Pearson correlation calculations are based on the assumption that both X and Y values are sampled from populations that follow a Gaussian distribution, at least approximately (GraphPad, 2002). When two variables vary together, a high correlation between these variables occurs. A significant correlation is characterized by a p-value of less than 0.05.

This correlation analysis was carried out on all parameters studied, following blind deconvolution. When performing correlation on these parameters as a function of depth, the

range of data was limited to the range corresponding to the top of the sample to the peak value of the parameter observed within the volume. Correlation analysis of various parameters to particle size was performed after removing two outlying points. This will be addressed further in section 3.2.8.

3. RESULTS & DISCUSSION

3.1. *Qualitative Results*

3.1.1. Crystallization in a Thin Film (2-Dimensional, Confined Space)

When viewed under cross-polarized light, solid crystal mass was observed in the form of bright distributions of particles. The anisotropic nature of solidified crystals (seen as various shades of white and grey) differentiates them from the isotropic liquid phase (which remains stark black in colour) (McCrone et al., 1978; Robinson & Bradbury, 1992).

Greyscale micrographs of the networks produced under thin film conditions are presented in Figure 3.1. Corresponding binary images, which were used for further quantitative analyses, are depicted in Figure 3.2.

Each nucleation event is believed to result in an individual primary particle of approximately 500nm in size (Narine & Marangoni 1999a; Marangoni, 2002). These particles then clustered to form significantly larger aggregates in the range of 100µm to 300µm (Fig. 3.1 & 3.2). On average, there were 10 large clusters within the field of view. When compared to images of focused planes of the same material growing in 3-dimensions (Fig. 3.3), it appears that the large clusters growing in 2D are similar in radial diameter and general 2D shape, but are more numerous than in any given focussed optical slice in 3D.

Figure 3.1: Polarized light micrographs (8-bit greyscale) of the model fat system crystallized for 15 minutes at 28°C in a thin film (approximately 20 μ m in thickness). Image names indicate slide number (1-3) and repetition (a-c). The width of each image corresponds to a length of 515 μ m.

Figure 3.2: Manually thresholded polarized light micrographs (binary) of the model fat system crystallized for 15 minutes at 28°C in a thin film (approximately 20 μ m in thickness). Image names indicate slide number (1-3) and repetition (a-c). The width of each image corresponds to a length of 515 μ m.

This observation intuitively suggests that the growth of clusters to these large sizes is strongly influenced by the volume available for growth. Since the thickness of the sample is limited to approximately 20 μm , spherulitic growth beyond a critical radius of 20 μm is hindered. Since these clusters continue to grow as crystallization progresses, the only possible subsequent circumstances would be: 1) continued, true spherulitic growth; 2) forced, continued lateral growth; or 3) some combination of the former two behaviours. Within the area of lipid crystallization, there is a void in the literature, and these issues have not been sufficiently investigated nor addressed.

Many crystallization conditions (such as slow cooling and/or crystallization at low degrees of supercooling) result in large clusters that exceed the sample thickness by several fold (de Man, 1961; de Man, 1964; Heertje et al., 1988; Grall & Hartel, 1992; Heertje, 1993; Fairley et al., 1995; Kawanari, 1996; Blaurock, 1999; Narine & Marangoni 1999c; Herrera & Hartel, 2000; Wright et al., 2000; Wright & Marangoni, 2002; Campos et al., 2002; Litwinenko et al., 2002; Marangoni & McGauley, 2003). These resulting structures have often been referred to erroneously in the literature as large “spherulites” when in fact simple geometric arguments would suggest that they are actually disc or plate-shaped with thicknesses of just a fraction of their lateral diameters. In theory and in practice, continued, unabated growth of large spherulites beyond the sample thickness is highly unlikely. The growth of large structures capable of physically “lifting” the cover slip and thereby increasing the overall sample thickness is not plausible. In practice, this behaviour would be easily detected, because conventional polarized light imaging would then be problematic. Difficulties in imaging would arise, since large clusters would exceed the depth of focus for the objective lenses used, and create multiple, focal planes. In practice, this is not observed

to any appreciable extent; however in some very specific, rare instances the growth of large clusters does increase the sample depth by a small amount (Singh, personal communication, 2002).

Despite the seemingly imminent influence of confinement on the growth of crystal networks, extensive empirical experimentation has demonstrated that valuable and credible information can still be harvested from images of networks growing under these conditions (de Man, 1961; de Man, 1964; Heertje et al., 1988; Grall & Hartel, 1992; Heertje, 1993; Fairley et al., 1995; Kawanari, 1996; Blaurock, 1999; Narine & Marangoni 1999c; Herrera & Hartel, 2000; Wright et al., 2000; Wright & Marangoni 2002; Campos et al., 2002; Litwinenko et al., 2002; Marangoni & McGauley, 2003). These analyses were carried out on the images of networks growing in 2D and on deconvolved 2D slices taken from 3D data sets and will be presented in section 3.2.

3.1.2. Crystallization in a Volume (3-Dimensions, Unconfined Space)

Raw Polarized Light Microscopy (PLM) at Multiple Imaging Planes

Thick samples, when viewed under cross-polarized light, demonstrated birefringence similar to that of thin films, but because crystallization occurred at various depths in the sample, any given optical plane within the volume contained both in and out-of-focus features. Features that fell above or below the focusing depth for a particular objective lens appeared as scattered light or haze. Micrographs depicting raw polarized light optical planes at various depths within the 207 μ m thick sample are presented in Figure 3.3.

At the first optical plane (Figure 3.3 – 0 μ m), which is the upper boundary, a significant amount of haze was observed, while no discernable features appeared to be in focus. As the motorized z-stage moved up, the focal plane moved incrementally deeper into the sample, and the hazy areas became progressively more crystal-like in appearance. It wasn't until a depth of approximately 40 μ m that features became in-focus. However, while some features appeared in focus, additional features that presumably lay at deeper points in the sample still contributed haze (see Figure 3.3 – 41.4 μ m). Stepping further into the sample, some of the larger clusters remained in focus at multiple focal planes, indicating that these features had geometries that spanned the z-axis. In other words, aggregated crystal masses were of sufficient size and appropriate shape to occupy 3-dimensional space. When stepping further into the sample, features in various XY positions appeared more in and out of focus in the z-direction, until a point deep within the sample where, similar to shallow depths in the sample, features no longer in focus were present (see Figure 3.3 – 193.2 μ m). Beyond this depth, an appreciable amount of haze was again present. The presence of great amounts of haze at the top and bottom of the sample made it difficult to conclusively determine whether material was crystallizing on the top and bottom glass surfaces, or whether all mass was centralized in the middle of the volume confines.

Figure 3.3: Raw polarized light microscopy images of the 207 μ m thick sample. Shown here is every tenth slice, which represents a step size of 13.8 μ m into the sample. The actual experiment contains 150 images at 1.38 μ m increments. The width of each image corresponds to a length of 515 μ m.

After stepping through the sample from top to bottom, a “mentally mapped” rendering of the network could be predicted. The imaged volume consisted of six large clusters of crystalline material (almost half the number of clusters observed in thin film growth), each having different centres in 3D space (Figure 3.4D). Some of these 3D clusters appeared to be of similar diameter to those disc-shaped clusters observed in thin film crystallization (Figure 3.1), however much of the crystallized mass was in the form of one very large branched cluster (Figure 3.4D – 1). This large 3D cluster was not generally of spherical shape, but was larger in XY dimensions (about 300 μm at its greatest length) than in its depth in the Z dimension (about 100 μm at its greatest length).

The presence of haze from planes above and below each optical slice also confounded the identification of the outer edges of the large clusters of particles. Moreover, small particles that made up the larger clusters were also difficult to identify discretely. The aim of deconvolution was to remove haze from out of focus planes, and therefore, improve this situation in order to be able to observe features and their locations with greater accuracy.

Nearest Neighbours Deconvolved Images of Multiple Planes

All 150 slices in the stack were deconvolved by the nearest neighbours (NN) algorithm. Every 10th slice within the stack (at 13.8 μm increments in depth) is shown in Figure 3.5 and their corresponding thresholded features shown in Figure 3.6. These images, when compared to their corresponding original raw PLM images (Figure 3.3), demonstrate a small degree of success in terms of haze removal and feature sharpening.

Figure 3.4: 2-dimensional projections of 3-dimensional stacks of the 207 μm thick sample: A) raw polarized light, with D) major features labelled; B) following deconvolution by the nearest neighbours algorithm, and E) subsequent thresholding; and C) following deconvolution by the adaptive blind algorithm and F) subsequent thresholding. Projections were obtained by the brightest point merge technique. The width of each image corresponds to a length of 515 μm .

When comparing the uppermost slice in the NN deconvolved volume to the raw PLM image at the same Z-position much but not all out of focus haze was removed. When

progressing deeper into the stack the presence of less haze was still noted at depths that did not include in-focus material (Figure 3.5 - 13 μ m to 55.2 μ m). Some of this haze was retained upon subsequent thresholding (Figure 3.5 - 13 μ m to 55.2 μ m). Marked improvements in the ability to observe discrete crystallites could be noted at depths that included in-focus features (Figure 3.5 - 69 μ m to 165.6 μ m) however some residual haze was also retained upon thresholding for feature isolation (Figure 3.5 - 69 μ m to 165.6 μ m). In slices at greater depths yet, where no in-focus features existed (193.2 μ m – 207 μ m) significant haze was observed but a slight improvement over the raw slices was observed in all slices.

Qualitatively, NN deconvolution was successful in removing only a portion of the out of focus haze present, and therefore wasn't as efficient as anticipated.

Figure 3.5: Polarized light microscopy images of the 207 μm thick sample following deconvolution with the nearest neighbours algorithm. Shown here is every tenth slice, which represents a step size of 13.8 μm into the sample. The actual experiment contains 150 images at 1.38 μm increments. The width of each image corresponds to a length of 515 μm .

Figure 3.6: Thresholded polarized light microscopy images of the 207μm thick sample following deconvolution with the nearest neighbours algorithm. Shown here is every tenth slice, which represents a step size of 13.8μm into the sample. The actual experiment contains 150 images at 1.38μm increments. The width of each image corresponds to a length of 515μm.

Blind Deconvolved Images of Multiple Planes

The raw PLM stack was deconvolved by the blind algorithm, and every 10th slice within the stack (at 13.8 μ m increments in depth) is shown in Figure 3.7 and their corresponding thresholded images in Figure 3.8. These images demonstrate a significant improvement in haze removal and feature sharpening when compared to their corresponding original raw PLM images (Figure 3.3), and demonstrate much better results than those arrived at by NN deconvolution (Figure 3.5). The presence of a small amount of focussed material on the top and bottommost slices may have been an indication that a small amount of the sample crystallized on the glass surfaces. Following deconvolution by the blind algorithm, no detectable haze was present in all slices within the stack. For this reason, the images deconvolved by the blind algorithm are believed to be most representative of the actual crystal network.

Another observation that could be made from qualitative examination of the micrographs was the apparent presence of more crystal mass in the lower half of the volume, which may have resulted from an uneven distribution of cooling of the sample, and can be attributed to the construction of the cold stage apparatus. This hypothesis will be addressed in more detail in a following discussion on quantitative results (section 3.2.1).

Figure 3.7: Polarized light microscopy images of the 207 μm thick sample following deconvolution with the adaptive blind algorithm. Shown here is every tenth slice, which represents a step size of 13.8 μm into the sample. The actual experiment contains 150 images at 1.38 μm increments. The width of each image corresponds to a length of 515 μm .

Figure 3.8: Thresholded polarized light microscopy images of the 207 μm thick sample following deconvolution with the adaptive blind algorithm. Shown here is every tenth slice, which represents a step size of 13.8 μm into the sample. The actual experiment contains 150 images at 1.38 μm increments. The width of each image corresponds to a length of 515 μm .

3.1.3. 2-Dimensional Projections of a 3-Dimensional Volume

Raw PLM 2-Dimensional Projection

The 2-dimensional projection of the 3D data set provided a means of observing all of the network features (Figure 3.4A, D). Since this technique was a projection, all information regarding the 3rd dimension was lost, however, it allowed us to see all of the network features on one representative, collapsed 2D image. Since this projection contained the brightest pixels at all XY coordinates within the full raw PLM stack, it retained much of the haze that was described earlier. This haze contribution rendered the image unfit for reliable thresholding and quantitative analyses since any thresholding value chosen either retained much haze, or completely eliminated pertinent structures.

Examination of the 2D projection confirmed the presence of six large clusters of crystals, which are identified in Figure 3.4D. Comparison of the raw PLM 2D projection of the 3D volume to images of the same material crystallizing under the same cooling conditions in a thin film (Figure 3.1) demonstrate major differences in overall network structure and cluster properties. Crystal growth in unconfined space appeared to result in larger, less numerous crystal aggregates compared to the smaller, more numerous crystals resulting from growth in a thin film. This observation corresponds well to expectation: the same amount of material crystallizing in an unconfined space would have the innate ability to grow to larger proportions. In addition, the rate of heat transfer in a thin film would be greater than that of a larger sample. This would potentially lead to enhanced nucleation, which may have lead to the formation of a higher number of clusters. Also, within a thin film, crystallization of clusters with centers at greater distances from one another would be

promoted by highly localized heat evolution occurring during the phase transition from liquid to solid. This is likely the cause for a greater number of clusters observed in the thin film compared to less, larger clusters that were present in the volume sample.

A final qualitative observation lay in the amount of apparent mass in both scenarios. Even though the 2D projection may result in merging of mass at similar XY coordinates but varying depths, the overall mass occurring appears to be similar to the amount of mass observed in thin films under the same crystallization conditions. This hypothesis will be addressed quantitatively in section 3.2.1.

Deconvolved 2-Dimensional Projections

2-Dimensional projections of 3D image stacks following deconvolution by the nearest neighbours and blind algorithms are presented in Figures 3.4B and 3.4C respectively. Deconvolution and subsequent 2D projection revealed a small degree of haze removal by the nearest neighbours technique. While some features were enhanced, the presence of residual haze still confounded the ability to clearly identify small crystals within the larger clusters. This is more evident when examining the thresholded image in Figure 3.4E.

Following blind deconvolution, on the other hand, the 2D projection shows a distinct improvement in the ability to clearly identify discrete crystals, since most, if not all of the haze due to scattering was removed (Figures 3.4C, F). Examination of the FFT power spectrums of 2D projection images revealed the relative amounts of poorly focussed or hazy features (Figure 3.9). The cross pattern observed in the power spectrum of the raw 2D projection indicates significant presence of haze (Figure 3.9A). Following NN deconvolution, the relative amount of haze is reduced, and can be seen as a narrowing of the

cross pattern in its power spectrum (Figure 3.9B). Finally, the high degree of haze removal by the blind deconvolution technique results in just a faint trace of the banding in the power spectrum (Figure 3.9C). This qualitative examination of the power spectrum serves as a semi-quantitative proof of the relative efficiencies of the two deconvolution algorithms used.

Figure 3.9: Forward Fourier transforms of 512x512 cropped areas of 2-dimensional projections of 3-dimensional stacks of the 207 μ m thick sample: A) raw polarized light, B) following deconvolution by the nearest neighbours algorithm, and C) following deconvolution by the adaptive blind algorithm.

3.1.4. 3-Dimensional Volume Renderings

Because 3-Dimensional volume renderings cannot be fully appreciated and evaluated in the form of 2D printed images, they are included in digital format on the DVD in the Appendix. To access them, the HTML file entitled “index” should be opened in any web browser on a Macintosh or PC computer capable of viewing AVI file types.

By viewing the 3D rotating movie files, comparison of the raw PLM rendering (“raw_3Drender.avi”) to those created from NN (“8n_neigh_3Drender.avi”) and blind deconvolution (“blind_3drender.avi”) reveal dramatic differences in the level of efficiency of haze removal and improved feature recognition. As was the case in the examination of

deconvolved 2D slices and 2D volume projections, the nearest neighbours algorithm removed only a small portion of the out-of-focus haze. The blind algorithm, on the other hand, demonstrated a high degree of haze removal and feature sharpening.

Qualitative information about the structure of the crystal network in 3D space can also be taken from these renderings. For example, the localization of almost all of the crystal mass within the center of the volume, and not at the upper and lower boundaries is clearly evident. The locations of the large clusters relative to the embedding space and relative to other clusters in the volume can also be qualitatively observed.

One observation of particular significance can be made when observing the network in the YZ plane (by pausing the rendering video, this plane can be observed without the burden of motion). Crystals appear to be somewhat stretched in the z-direction. This may be due to a number of causes including: an artefact of the 3D rendering process; improper calibration of the z-stage; or crystals actually tend to grow in this manner. Calibration of the z-stage is far from trivial, but might rule out any of the proposed causes for this observed stretching behaviour. One possible calibration procedure is discussed in more detail in the section on recommendations for future research (section 6).

3.2. Quantitative Results

3.2.1. Area Fraction (% black)

The amounts of crystal mass present, determined by computing the area fraction of binary images of 2D slices from each deconvolved volume, their 2D projections, and the structures that resulted from growth in thin films are presented in Figure 3.10.

Figure 3.10: Area fraction (%) versus depth (μm) in a 207 μm thick sample. Every tenth slice (or 13.8 μm) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a 20 μm thin film sample are also included for comparison.

2D Slices of the 3D Volume

An immediately evident trend is observed for results of the area fraction of deconvolved stacks as a function of slice depth. In both cases, results suggest that there is a higher concentration of solid crystal mass in the middle portions of the volume, with progressively less mass residing in the upper and lower portions of the sample. These results

also suggest that crystal growth had not taken place to an appreciable extent at the upper and lower sample boundaries. This also suggests that the growth mode of the clusters is of a radial nature, with early crystallization events occurring centrally and subsequent growth occurring in all directions. As noted in section 3.1.2, many of the clusters were of nearly spherical geometry, but one predominant large cluster was significantly larger in XY plane than Z plane (Figure 3.4D-1) and contributed greatly to these measured results.

Another observed trend was the shift in peak mass to slightly below the middle depth. This shift in mass concentration to depths below center may mean that the earliest nucleation conditions of under-cooling and molecular compatibility were satisfied below center. This may also be supported by the hypothesis that this downward shift in mass localization may have been in part influenced by the nature of the cooling apparatus used. The construction of the Linkam cold stage is such that the greatest driving force for cooling may be located directly beneath the sample, creating a gradient in heat transfer that is in theory greater toward the bottom of the sample. Liquid nitrogen is pumped into the stage apparatus, and is circulated through a high heat capacity (copper) block that supports the sample slide. As a result, the overall sample temperature may not be evenly distributed in the z-direction, and enhanced conditions for nucleation and crystal growth may be created in a location below the center of the sample. It is also possible that sedimentation due to gravity occurred prior to imaging.

Comparison of the results for the two deconvolution algorithms used yielded quantitative results that confirmed qualitative observations. Since more haze was removed by the blind technique, the area occupied by remaining thresholded features was slightly lower in all slices studied. However, the peak area occurred at the same depth in both cases,

indicating that both methods result in highly similar indications of mass relative to depth. The exception to this finding is that the residual haze in the NN data resulted in a gross overestimation of the amount of crystal mass present in the uppermost and lowermost slices (on the glass surfaces). On the other hand, values of near zero area fraction at the top and bottom slices following blind deconvolution correspond to the visual inspection of slices and belief that just a very small amount of crystal was actually present at these locations.

A significant linear correlation coefficient between area and depth ($r=0.98$, Table 3.1) indicated that as the sample was analyzed from the top to the peak value (near the middle) more mass was progressively present.

2-Dimensional Projections of 3D Volumes

Comparison of the 2D projections following both deconvolution methods revealed no appreciable differences in the area fraction upon thresholding. Since the majority of haze results from scattering directly above crystal mass at lower depths, much of the haze retained following the NN deconvolution would have been obscured by the lower laying crystal mass. Therefore, the haze in the 2D projection did not contribute to an increase in the area fraction upon thresholding. Both 2D projections values, however, are greater than the peak value within the volume because the projections include the contribution of all the clusters present. Also important to note is that the area fraction for the 2D projections are not equal to the cumulative contribution on the slices that make up the 3D stack. This is due to two factors: 1) the occurrence of masking and merging of features from separate clusters that share XY coordinates but are located in various different locations in the Z direction, and 2) some

features within clusters extend into multiple planes in the Z-direction. Visual examination and interpretation of the 3D renderings in the Appendix DVD support both of these claims.

Thin Film Conditions

The area fraction of crystal networks that resulted from growth under confinement in thin films are also shown in Figure 3.10. After observing these networks (Figure 3.1) and knowing that the clusters are forced to grow laterally, the fact that they possess a higher area fraction relative to any given 2D slice from a volume set and relative to the 2D projections can be expected. Although differences in the amount of crystalline material that resulted in both the volume and thin film samples were assumed to be negligible, the nature of the growth of the material differed and was detectable via differences in the area fraction.

3.2.2. Fractal Dimension (D_f) – Degree of Order and Fill

Fractal analyses of polarized light micrographs revealed characteristics of the crystal networks growing in a volume and in thin film conditions. In particular, information pertaining to the spatial distribution of mass and in the network and the degree of fill (via D_f and D_b) was obtained. These analyses also yielded new-found experimental evidence related to fractal scaling theory in 2-dimensional and 3-dimensional Euclidean space.

The particle-counting fractal dimension (D_f) is sensitive to both the degree of order and fill displayed by the particles that make up the network (Narine & Marangoni, 1999a,b,c; Marangoni, 2000; Marangoni, 2002). As stated previously, a high value for D_f is associated with a network that results from a more space-filling network and/or more orderly distribution of particles in space, and often is the result of slow cooling or cooling to lower

degrees of supercooling for example (Narine & Marangoni, 1999a,b,c; Marangoni, 2000; Marangoni, 2002; Litwinenko et al., 2002). On the other hand, lower relative values for D_f can be expected when particle distribution is more disorderly in nature, and/or less space-filling, such as under faster cooling or cooling to a higher degree of supercooling. Empirically, D_f is believed to be more sensitive to order than to space-filling properties (Marangoni & Awad, personal communication, 2003). This analysis has been used extensively in thin film studies, and only now is being used to examine volumes. Fractal dimension determinations by the particle-counting method of 2D slices of 3D volume stacks, 2D projections of 3D volumes, and 2D thin film conditions are depicted in Figure 3.11.

Figure 3.11: Particle-counting fractal dimension (D_f) versus depth (μm) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

2D slices of the 3D volume

Much like the trends observed for the area fraction for 2D slices of the 3D volume, D_f values were highest ($D_f \sim 1.6$) at depths in more central portions of the sample. This experimental

value corresponds well with the predicted value of particle-cluster diffusion-limited aggregation ($D=1.7$, $d=2$) by computer simulation and is an indication of the growth mode of the large clusters of particles (Viscek, 1992; Rothchild, 1998). This also intuitively makes sense since the slices that demonstrated this value of 1.6 consisted primarily of a cross section of one large cluster. This also indicates that the spatial distribution of mass in this general location was more orderly compared to the upper and lower regions of growth where values were much lower ($D_f=1.10-1.40$). Intuitively, if the large clusters are growing in a somewhat spherical fashion (section 3.1.2) then it stands to reason that the distribution of mass near the centers of such clusters would be of higher order, simply because they have had more time and ability to set. Changes in crystal habit that involve molecular transitions to more stable polymorphs can occur within minutes of the onset of crystallization (Kellens et al., 1992; van Malssen et al., 1996; Breitschuh & Windhab, 1998; ten Grotenhuis et al., 1999; van Langevelde et al., 2001; Marangoni & McGauley, 2003), but transitions to more stable structures at the mesoscale level are not as easily demonstrated. Since crystallization in the case of this study was conducted at a relatively low degree of supercooling, it is conceivable that diffusion and molecular mobility may be high enough to allow for restructuring. Also, some experimental evidence from literature suggests that in thin films, large discs formed by slow cooling or cooling to low degrees of supercooling can result in more compact or orderly crystal structure in their interiors relative to their outermost regions (Marangoni & McGauley, 2003).

The degree of order was found to be significantly correlated with depth, when analyzed from the top of the sample to the peak value, below the middle of the sample

($r=0.92$, Table 3.1). This would indicate that the sample was more full and/or more orderly as a function of depth in this range.

2-Dimensional Projections of 3D Volumes

The values of D_f for the 2D projections were similar following deconvolution by both techniques (Figure 3.13). This indicates that differences in the degree of haze removal due to the two deconvolution methods, when presented as 2D projections, did not result in a detectable difference in spatial distribution of mass. An experimental D_f value of 1.63 for the blind 2D projection corresponds well with the predicted value for cluster-cluster diffusion-limited aggregation ($D=1.7-1.8$, $d=3$) (Viscek, 1992; Rothchild, 1998). This intuitively makes sense, since we are observing a projection of a group of clusters in 3D embedding space that could quite conceivably be part of a larger cluster. More interesting, however, is the finding that the experimental particle-counting fractal dimension of the 2D projection of the 3D volume ($D_f=1.63$) corresponds to the peak value observed within the 2D slices of the 3D volume ($D_f=1.66$). This result provides new-found experimental evidence for fractal scaling properties that have long been theorized but have not to date been demonstrated in the literature. Theory states, as a “rule”, that an object in 3-dimensional Euclidean embedding space, that has a fractal dimension less than 2, when projected onto a 2-dimensional Euclidean plane, will retain its fractal dimension (Vicsek, 1992). This result for the system studied offers preliminary evidence in the form of an actual experimental system.

Also, it is believed that if the object in 3D space has a dimensionality of greater than 2, when projected onto a 2D plane, will completely fill that space and the fractal dimension

would collapse to a value of 2 (Vicsek, 1992). This fractal scaling property suggests that the distribution of mass in unconfined volumes has a fractal dimension less than 2, since when collapsed to 3D it is not space-filling. This finding does not correspond, however, to rheological determinations of D_f , in which case values between 2 and 3 are the norm, perhaps due to much higher degrees of supercooling used for rheological methods. This may, on the other hand, suggest that perhaps the growth of networks prepared for rheological determinations of D_f are truer volumes, and that perhaps a network grown in a sample of $207\mu\text{m}$ in thickness is still influenced by confinement. Since large clusters with radial diameters greater than the total sample thickness were observed, a small yet substantial effect of confinement may still occur, and confound the fractal dimension determinations. Furthermore, it may be possible that rheological methods are sensitive primarily to particle cluster interactions (which correspond to a $D=2.5$ in 3D space) (Vicsek, 1992; Rothchild, 1998) while 2D projection image analysis techniques are confounded by the inability to disseminate the cross over between particle-cluster and cluster-cluster aggregation processes.

Thin Film Conditions

The particle-counting fractal dimension of networks grown in thin film conditions resulted in a characteristic value of 1.94. This result, when compared to the values obtained for the 2D projections of slices in 3D space offers some fresh perspective. In this instance, new-found information pertaining to the effects of confined growth is demonstrated. The much higher value for D_f resulting from growth in a thin film ($D_f=1.94$) relative to the peak value and 2D projection values of deconvolved stacks ($D_f \sim 1.60$) and predicted values by

simulation ($D_f = 1.7$) provides further evidence that growth under confinement is in fact creating conditions for more orderly, nearly homogeneous network formation ($D=2$).

3.2.3. Fractal Dimension (D_b) – Degree of Fill and Order

Empirically, the box-counting fractal dimension (D_b) is sensitive primarily to the degree of fill or space occupancy of particles that make up the network (Marangoni & Awad, personal communications, 2003; Marangoni & McGauley, 2003). A high value for D_b is associated with a network that results from a more space-filling network, and often is the result of fast cooling or cooling to higher degrees of supercooling (Marangoni & McGauley, 2003). On the other hand, lower relative values for D_f can be expected when the network is less space-filling, such as under slower cooling conditions or cooling to a lower degree of supercooling (Marangoni & McGauley, 2003). Little in the way of experiment evidence has demonstrated conclusively that D_b is influenced by the spatial distribution of mass of fat crystal networks. Fractal dimension determinations by the box-counting method of 2D slices of 3D volume stacks, 2D projections of 3D volumes, and 2D thin film conditions are depicted in Figure 3.12.

2D Slices of the 3D Volume

Analyses of the blind deconvolved 2D slices of the 3D volume revealed increasing values for D_b with increasing depth, up to a peak value ($D_b=1.66$) which occurred below the middle of the sample ($r=0.96$, Table 3.1). Much like was revealed by D_f , the box-counting fractal dimension D_b corresponds well to predicted values arising from diffusion-limited

particle-cluster aggregation ($D=1.7$, $d=2$). This also indicates a tendency for a higher degree of space-filling near the center of the sample relative to the upper and lower regions.

Figure 3.12: Box-counting fractal dimension (D_b) versus depth (μm) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

This also corresponds to results for area fraction (section 3.2.1, Figure 3.10), and with qualitative observations described in section 3.1.2. Once again, the shift in peak value to a depth slightly below center may be attributed to the nature of the apparatus used for temperature control. Further evidence of the failure of nearest neighbours deconvolution to remove all scattered light is clearly observed here, since in the upper and lower regions of the volume, where residual haze was present, space-filling tendencies are detected in regions where little to no crystal mass was actually present.

2-Dimensional Projections of 3D Volumes

Values of D_b for the 2D projections were similar following deconvolution by the NN and blind methods ($D_b=1.66$). This indicates that the differences in the degree of haze

removal due to the two deconvolution methods, when presented as 2D projections, did not result in a detectable difference in the degree of fill in the network. Since the majority of haze results from scattering directly above crystal mass at lower depths much of the haze retained following the NN deconvolution would have been hidden amongst the lower laying crystal mass in the 2D projection, and hence would not contribute enough to heighten space-filling tendencies upon thresholding. This also is observed qualitatively as slight blurring of small crystals that make up the larger clusters (Figure 3.4B).

Once again, the fractal dimension of the 2D projection of the 3D clusters in the volume ($D_b=1.66$) corresponds well to predicted values that arise from diffusion-limited cluster-cluster aggregation ($D=1.7-1.8$, $d=3$) (Viscek, 1992; Rothchild, 1998). These values are very similar to the peak value observed within the 2D slices of the 3D volume. This result once again provides further novel experimental evidence for the theorized rule that an object in 3D space that has a fractal dimension less than 2, when projected onto a 2D plane, retains its fractal dimension (Vicsek, 1992).

Thin Film Conditions

The box-counting fractal dimension of networks grown in thin film conditions resulted in a characteristic value 1.67. This result shows strong agreement with the values obtained for the deconvolved 2D projections of slices in 3D space ($D_b=1.66$) despite differences in area fraction (23.5% in thin films as opposed to 19.3% in deconvolved 2D projections). This finding suggests that perhaps other network characteristics in addition to space-filling tendencies may have an influence on D_b . This will be further addressed in section 3.2.9.

3.2.4. The Relationships between Fractal Dimensions and Area Fraction

Since D_f and D_b are both believed to be sensitive to the degree of fill in the network (more is the case for D_b), plots of these parameters as a function of area fraction were examined (Figures 3.13 and 3.14).

Figure 3.13: Particle-counting fractal dimension (D_f) versus area fraction (%) in a 207 μ m thick sample. Every tenth slice (or 13.8 μ m) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a 20 μ m thin film sample are also included for comparison.

Figure 3.14: Box-counting fractal dimension (D_b) versus area fraction (%) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

The particle-counting fractal dimension D_f did not display a direct linear relationship with area fraction, although linear correlation analysis of the blind deconvolved data indicated a significant positive relationship ($r=0.79$) between these two variables (Figure 3.13 and Table 3.1). This supports our understanding that D_f is influenced in part by the amount of crystalline mass in the network, and in part the spatial distribution of mass (Narine & Marangoni, 1999a,b,c; Marangoni, 2000; Marangoni, 2002; Litwinenko et al., 2002). Once again, it can be observed that 2D projections of the 3D slices of the volume retain their peak value, and the much higher values for D_f and area fraction in thin films suggest that cluster growth is affected by confinement.

The box-counting fractal dimension D_b displayed a linear relationship with area fraction, and linear correlation analysis of the blind deconvolved data indicated a significant positive relationship ($r=0.96$) between these two variables (Figure 3.14 and Table 3.1). This

lends further novel support to our belief that D_b is more highly influenced by amount of crystalline mass in the network than D_f ($r=0.71$) (Marangoni & Awad, personal communication, 2003). Since the 2D projection and thin film values of D_b remain constant despite having heightened area fractions, this suggests that D_b is in part influenced by some factor other than the quantity of crystalline mass. This will be further addressed in section 3.2.9.

3.2.5. Number of Particles (N)

2D Slices of the 3D Volume

The number of particles, as defined in section 3.2.1, was computed for all micrographs that made up the volume element (Figure 3.15). Results following blind deconvolution displayed trends similar to those found in area fraction, with a gradual increase to a peak near the middle of the sample followed by a subsequent decrease. Linear correlation analysis of particle number revealed a significant positive relationship to both depth (from the top of the sample to the peak within, $r=0.96$) and area fraction ($r=0.98$), as would be expected (Table 3.1). The granular morphology of the crystallites that make up the clusters, in this case, means that a higher number of particles results in more overall mass (detected as area fraction), which is shown to occur near the middle of the sample (Figure 3.10).

Figure 3.15: Number of particles (N) versus depth (μm) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

The much higher number of particles observed following deconvolution by the NN technique is yet another consequence of incomplete haze removal by the NN technique. Some of this residual haze was retained following thresholding (Figure 3.5), resulting in small artefactual features which subsequently resulted in higher than actual particle counts for NN slices, especially near the upper portion of the sample. A return to the thresholded images in the upper slices reveals clearly the presence of this artefact (Figure 3.6 – $000\mu\text{m}$ to $41.4\mu\text{m}$).

2-Dimensional Projections of 3D Volumes

The number of particles observed in 2D projections of the 3D volumes reveals a number of qualities worthy of note. Firstly, the values of N for the projections are not equivalent to the cumulative contribution of the slices that make up the stack, but in fact are

even lower than the values observed with a given slice (near the middle, for example). This is easily explained by commenting on the nature of the projection method. Inherent to the brightest point merge projection technique, loss in feature isolation or detail occurs when features are of similar brightness in a particular shared XY coordinates but originate from different Z coordinates. The consequence of this loss, which does inevitably occur (and would with any alternative projection technique chosen), is the masking and merging of features, and a value for N that is less than that of the 3D volume. A much higher number of particles present in the 2D projection following NN deconvolution (relative to the blind counterpart) was again attributable to partial retention of haze contribution upon thresholding and can be observed in the 2D projection image (Figure 3.4E).

Thin Film Conditions

The number of particles observed in thresholded images of thin films is similar to that of the 2D projection following blind deconvolution. Although, in theory, the thinner sample would result in a network with a greater number of particles, the effect of confinement (which earlier was shown to increase order) may also result in denser, more compact clusters. The higher density of small crystals that make up the large clusters may result in the inability to resolve individual crystallites. Once again, this behaviour, and its affects on quantitative image analysis, is not well established in the literature. This behaviour is observed qualitatively in micrographs (Figures 3.1 and 3.2).

3.2.6. The Relationships between Number of Particles (N) and Area Fraction (%)

The number of particles that make up the network following blind deconvolution is significantly correlated with the amount of space occupied by the particles ($r=0.98$, Table 3.1) and is depicted in Figure 3.16. Artefacts resulting from incomplete haze removal after NN deconvolution (especially in the uppermost and lowermost slices) result in unreliable data for the number of particles and area fraction (more so N). Projections and thin films display a lower N value due to same reasons previously described in section 3.2.4.

Figure 3.16: Number of particles (N) versus area fraction (%) in a 207 μ m thick sample. Every tenth slice (or 13.8 μ m) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a 20 μ m thin film sample are also included for comparison.

3.2.7. The Relationships between Number of Particles (N) and Fractal Dimensions (D_f and D_b)

The number of particles that make up the network following blind deconvolution is significantly correlated with the degree of fill and order displayed by the network (D_b : $r=0.97$, D_f : $r=0.79$, Table 3.1) and is depicted in Figures 3.17 and 3.18. This would be expected, since both D_b and D_f are both highly correlated with area fraction as well. Once again, artefacts resulting from inefficient haze removal by NN deconvolution resulted in unreliable data for the number of particles and D_b , especially relevant in the upper and lower regions of the sample. Projections and thin films display a reduced N value due to same reasons previously described (section 3.2.4.). A constant D_b value in these cases demonstrates the influence of more than simply fill, and will be addressed in section 3.2.9.

Figure 3.17: Particle-counting fractal dimension (D_f) versus the number of particles (N) in a 207 μm thick sample. Every tenth slice (or 13.8 μm) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a 20 μm thin film sample are also included for comparison.

Figure 3.18: Box-counting fractal dimension (D_b) versus the number of particles (N) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

3.2.8. Microstructural Element Size (MES)

In all cases, particle distribution analysis resulted in MES values that corresponded to visual examination of slices within the volume stack. However, in two instances (blind results for slices at $41.4\mu\text{m}$ and $55.2\mu\text{m}$, see Figure 3.8) a very low number of large clusters were present, and were not representative of the particles observed in the volume as a whole. For this reason, these two points skewed results and were excluded from correlation analysis.

MES and Depth

The size of particles that make up the network following blind deconvolution was significantly correlated with the depth within the sample from the top to the location at peak MES ($r=0.87$, Table 3.1) and is depicted in Figure 3.19. This indicated that mean size of

particles making up the network increased as a function of depth in the range studied, and may indicate that growth originated near the center and at the time of imaging those particles near the center had simply had more time to grow to larger proportions. Once again, residual haze artefacts resulting from NN deconvolution led to unreliable data for the mean size of particles. Projections and thin film display increased MES due to masking and merging of particles as previously described in section 3.2.4.

Figure 3.19: Microstructural element size (μm^2) versus depth (μm) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

MES and Area Fraction (%)

The size of particles that made up the network following blind deconvolution was significantly correlated with the area fraction ($r=0.86$, Table 3.1) and is depicted in Figure 3.20. This indicates that slices composed of larger particles also covered more area, and once again corresponds to the belief that the earliest and more progressive crystal growth occurred near the center of the sample. Results from NN deconvolution do not give reliable data for

the size of particles due to residual haze. Projections and thin film display increased MES and area fraction due to same reasons previously described in section 3.2.4.

Figure 3.20: Area fraction (%) versus microstructural element size (μm^2) in a 207 μm thick sample. Every tenth slice (or 13.8 μm) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a 20 μm thin film sample are also included for comparison.

MES and Degree of Fill (D_b)

The size of particles that make up the network following blind deconvolution is significantly correlated with the degree of fill ($r=0.81$, Table 3.1) and is depicted in Figure 3.21. Artefacts resulting from NN deconvolution do not give reliable data for the size of particles. Projections and thin film display increased MES while D_b remains constant due to same reasons previously described.

Figure 3.21: Microstructural element size (μm^2) versus box-counting fractal dimension (D_b) in a $207\mu m$ thick sample. Every tenth slice (or $13.8\mu m$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu m$ thin film sample are also included for comparison.

MES and Number of Particles (N)

The size of particles that make up the network following blind deconvolution is significantly correlated with the number of particles in the network ($r=0.85$, Table 3.1) and is depicted in Figure 3.22. Artefacts resulting from NN deconvolution do not give reliable data for the size nor the number of particles. Projections and thin film display increased MES and while N is less than the max in the volume due to same reasons previously described (masking and merging, and confinement respectively).

Figure 3.22: Microstructural element size (μm^2) versus number of particles (N) in a $207\mu\text{m}$ thick sample. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack (nearest neighbours and blind algorithms) was analyzed. 2D projections (by brightest point merge) of each 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

3.2.9. Standard Deviation of Mean Nearest Neighbour Analysis as an Indicator of Order

The influence of area fraction or degree of fill on the fractal dimensions D_f and D_b was demonstrated by high correlation with area fraction (section 3.2.5), however theory predicts that the fractal dimensions D_f and D_b are also influenced by the degree of order in the distribution of mass (Marangoni, 2002; Litwinenko et al., 2002). Therefore, an attempt was made to reveal an alternative indicator of order from polarized light microscopy. This was performed by examining the variation (or standard deviation) in the determination of mean nearest neighbour distance (NND). Figure 3.23 demonstrates the relationships between the standard deviation of the mean NND and D_f and D_b following blind deconvolution. In both cases, significant negative correlation coefficients were found (D_f : $r=-0.72$, and D_b : $r=-$

0.73, Table 3.1). This means that a higher fractal dimension occurs in images of the network that show lower variation in the distribution of particles, which is analogous to a more orderly particle distribution. Conversely, a low fractal dimension is associated with a larger variation in NND, which indicates a more disorderly particle distribution. Both fractal dimensions thus demonstrate a dependence on both order and fill.

Figure 3.23: The standard deviation of the mean nearest neighbour distance (NND) as a function of the fractal dimensions A) D_f , and B) D_b following blind deconvolution. Every tenth slice (or $13.8\mu\text{m}$) within the deconvolved 3D stack was analyzed. The 2D projection (by brightest point merge) of the 3D stack and a $20\mu\text{m}$ thin film sample are also included for comparison.

The differences observed for the D values for thin film growth provide further evidence for the relative contributions of order and fill. While the D_f value is much higher in thin films than in a volume (and thus demonstrates the effect of confinement on order and fill), the D_b value is not elevated in thin film conditions (despite having a higher area fraction and higher apparent order). This suggests that the box-counting method is not as sensitive to order (relative to fill) in thin film conditions. This finding corresponds to previous studies where D_f and D_b have been found to be inversely proportional (Marangoni & Awad, personal communications, 2003).

3.2.10. Pearson Linear Correlations of Blind Deconvolved Data

Table 3.1 highlights the Pearson linear correlation coefficients for various network parameters determined by image analysis of the 2D slices of a 3D volume following blind deconvolution. Correlations that are statistically significant are bolded. High correlation indicates that the variables studied vary well together (GraphPad, 2002), and are thus statistically associated. Significant correlation between most of the studied parameters was observed, and suggests a high level of inter-relatedness between network characteristics. The number and location of particles influences the size of the particles, which in turn influences the area fraction. The area fraction influences the fractal dimensions which are also correlated with the apparent degree of order in the spatial distribution of mass. These correlations may be system and condition specific, however. Further studies on various systems and crystallization conditions one of the many recommendations for future work (section 6).

Table 3.1: Pearson linear correlation coefficients for various network parameters determined by image analysis of polarized light micrographs of 2D slices of a 3D volume following blind deconvolution. Negative correlations indicate an inverse relationship. Correlations that are statistically significant are bolded.

	Depth**	Area Fraction	D_f	D_b	N	Standard Deviation of NND
Area Fraction	0.98					
D_f	0.92	0.79				-0.72
D_b	0.96	0.98	0.84			-0.73
N	0.96	0.98	0.84	0.97		
MES*	0.87	0.86	0.26	0.81	0.85	

Bolded: $p < 0.05$

*outliers removed

**from top of sample to peak value

3.2.11. Note on the Effects of Edges, Optics, Magnification, and Image Resolution on Fractal Dimensions

Edge Effects on Fractal Dimension Determination

Recent investigations into the effects of including and excluding of features that fall on the edges of the region of interest (ROI) in the particle-counting procedure have revealed important cautions that must be heeded in order to avoid artefacts (Marangoni, 2002; Litwinenko et al., 2002). Further artefacts also occur when the size of the ROI gets small. This issue will be addressed only briefly here.

Figure 3.24A depicts the results of the particle-counting method (both including and excluding features that touch the edges of each ROI) for an image of cocoa butter, courtesy of Tarek Awad. In this case, the ROI was iteratively reduced from 100% to 5% of the original image size. Figure 3.24B depicts results from the same image but only the ROIs

representing 100% to 35% of the original image size are included. The number of particles is plotted against the length of the ROI in a log-log fashion. The resulting slope in each case is an uncorrected D_f .

Figure 3.24: Log-log plots of the number of particles (N) versus the length of the box (L) used to determine the particle-counting fractal dimension D_f . In A) counts corresponding to regions of interest spanning 100% to 5% of the original image length are included, whereas in B) the region used is 100% to 35% of the original image length. Data courtesy of Tarek Awad.

Two artefacts related to the effects of counting features at the edges of the ROIs are observed. Firstly, the effect of the contribution of small boxes is a skewing of the D value to elevated values. This occurs because as the box size gets small, a tailing off occurs on the

log-log plot. Once the box sizes become very small, the length of the box sizes decreases faster than the areas of the ROIs. This is especially problematic if the features falling on the edges of the ROI are large, since they will be greatly included and excluded in their respective counts. For this reason, performing the counts on ROIs representing 100% to 35% of the original box size is more accurate.

Secondly, if the particles that touch the edge of each ROI are excluded from the counts, the resulting slope is elevated due to an over-exclusion. Conversely, if the particles on the edges of the ROI are all included in the counts, a lower than true value results because of an under-inclusion artefact.

Unfortunately, the image analysis macro used is not capable of counting the particles touching only two of the four sides of each ROI (an adjusted count). To compensate, the average of the D_f values obtained by including and excluding counts in the 100% to 35% range is taken. This is analogous to counting just those features touching two of the four sides of each ROI, eliminates the skewing effect of small boxes, and results in an accurate D_f value (Marangoni, 2002; Litwinenko et al., 2002). For the image data portrayed in Figure 3.24, the corrected D_f has a value of 1.70.

Effects of Optics, Magnification, and Image Resolution on Fractal Dimension

Determination

Previous to the recent implementation of the Leica system used for imaging thin films and the volume in this study (see section 2.1.2.) another older system was used. This older system consisted of a polarizing microscope (Olympus model BH, Tokyo, Japan), a CCD video camera (Sony model XC75), and 640x480 video capture board (Scion, Frederick, MD,

USA). These two systems differ in terms of the quality of the optics, the type of camera, the magnification used, and spatial resolution of the images acquired. It was therefore of interest to verify results of image analysis parameters, particularly the fractal dimensions D_f and D_b , from images obtained using both systems. Soon to be published microstructural data from a separate study on the same model fat system crystallized by the same cooling conditions in thin film form were obtained from images obtained on this older imaging system. These results are presented here as an indication of the effects of differences due to the use of two unique imaging systems.

Figure 3.25 depicts a representative thresholded thin film image acquired on both systems. The relative sizes of the images were kept constant in order to demonstrate the differences in the actual sampling size. Note the differences in the sampling sizes and feature appearance obtained by imaging using a 50X objective and pixel resolution of 1280x1024 (Figure 3.25A) and that obtained by using a 10X objective and pixel resolution of 640x480 (Figure 3.25B).

Quantitative results for the fractal dimensions D_f and D_b determined on both imaging systems are presented in Table 3.2. Despite the many differences in imaging scenarios, both

Figure 3.25: Representative thresholded polarized light micrographs from A) the more recent Leica microscope system, and B) the older Olympus microscope system. Images acquired on the Leica system have pixel dimensions of 1280x1024 and were taken using a 50X objective lens. Images on the older Olympus system were 640x480 and the 10X objective used.

fractal dimensions were statistically similar (verified by a t-test, assuming equal variances).

This comparison suggests that the image analysis parameters are not influenced by differences in imaging and that in both cases, the imaged field is within the region of structure that exhibits fractal scaling properties.

Table 3.2: Fractal dimensions D_f and D_b and their standard deviations determined on two different microscopes, using different magnifications, and image spatial resolutions ($n = 7$).

	Imaging System	
	Old	New
D_f	1.91 ± 0.13	1.94 ± 0.12
D_b	1.67 ± 0.03	1.67 ± 0.03

4. ASSUMPTIONS AND LIMITATIONS

Since the methods employed in this study were unique, a number of assumptions were made that warrant further discussion. Perceived limitations inherent to the methods used are also discussed in the section that follows.

4.1. Z-Stage Calibration

For all 3D imaging experiments conducted on the imaging system in our laboratory, it has been assumed that the z-stage motor is properly calibrated. This is of utmost relevance if absolute 3D morphological measurements are desired (which has not been the case to date). If the z-stage stepping motor is out of calibration, it can be assumed that the drift from calibration is consistent at all depths. A recommendation for z-stage calibration and one possible method for performing this calibration are addressed in section 6.1.

Standardization Attempt: Bright-field Versus Polarized Light Microscopy

Blind deconvolution of transmitted light bright-field (TLB) volumes has been recently developed (Holmes et al., 1995; Holmes & O'Connor, 2001). The same is not true, however, for volumes imaged using polarized light. In order to assess the validity of imaging volumes for deconvolution using transmitted polarized light, comparisons of the z-series of an object that could be viewed under both conditions was performed.

A cylindrical fibre (believed to be nylon) was cut into small fragments, and embedded in triolein (the same medium that made up the liquid fraction of the fat system studied). When viewed by TLB, the fragments appeared as semi-translucent rods, with a dark center, which is consistent with the appearance of nylon and other synthetic fibres (McCrone et al., 1967). When viewed under polarized light the outer layer of the fibre was birefringent, and the rod size and shape appeared to be the same as when viewed by TLB. Z-series were then taken on the sample field of view, using both TLB and PLM, with the same Z-increments. Examining the XY and ZX slices of the raw image stacks demonstrated that under both lighting conditions, the fibres features and dimensions remained unchanged. Images of the fibre can be observed in Figure 4.1. This finding validated the technique of 3D z-series imaging of PLM images, however, an apparent stretching of the object (when examining the ZX profile) may be an indication the z-stage is improperly calibrated (see section 6.1).

Figure 4.1: Images of a nylon fibre under A) transmitted brightfield and B) polarized light. The XY planal view, and the coronal (ZY) views are shown for each. The width of each image corresponds to a length of $515\mu\text{m}$.

4.2. The Use of Fluorescence Settings in Deconvolution Procedures

Since no commercially available software is designed to handle 3D images obtained under polarized light conditions, deconvolution was carried out assuming a fluorescence model.

In the case of nearest neighbours deconvolution (which did not perform very well), it is not possible to conclude that deficiencies in haze removal were due to differences in the input theoretical fluorescence based PSF and the actual PSF on the sample. Incomplete haze removal may have simply been a result of deficiencies in the nearest neighbours algorithm itself, since noise from multiple planes is added together (Wallace et al., 2001).

In the case of blind deconvolution, it can be safely assumed that the use of a fluorescence based theoretical PSF (which merely serves as an initial estimate of the experimental PSF) has no profound influence on the results of deconvolution. Inherent to the blind technique is the construction of an experimental PSF from the data itself (Krishnamurthi et al., 1992; Bhattacharyya et al., 1996; McNally et al., 1999; Wallace et al., 2001), in which case the use of a fluorescence-based PSF as an initial estimate seems to be appropriate based on the quality of the results.

4.3. Limitations Related to Properties of Fat Crystal Networks

While performing various preliminary experiments using samples of greater depth, to higher degrees of supercooling, and following extended crystallization times, it became

evident that there is an ill-defined limit to the amount of birefringent material that can be present that still allows for successful volume imaging. When an excessive amount of birefringent crystal mass is present (dictated by solid fat content), volume imaging is confounded because it becomes increasingly difficult to differentiate crystals and clusters of crystals in both the XY direction and the Z direction. This limitation means that various compromises in crystallization conditions may be required. Compromises in the depth may be required if higher degrees of supercooling are desired, for example. Imaging of a sample at an earlier stage of crystallization may allow for higher degrees of supercooling, faster cooling rates, or increased depth conditions to be employed. The limitations of the technique will be highly system specific, and hence cannot be addressed in great detail at this time (see section 6.2). At the time of imaging, the SFC of the model fat system in this study was approximately 20% (determined by pulsed nuclear magnetic resonance).

5. CONCLUSIONS

Preliminary results from volumetric imaging of fats by wide-field deconvolution microscopy with polarized light suggest that this novel method is feasible. Deconvolution employing an adaptive blind algorithm is more suited to this technique than is a nearest neighbours algorithm because the blind method obviates the need to measure the point spread function under experimental conditions. Blind deconvolution also outperforms the nearest neighbours technique in terms of out-of-focus haze removal and feature isolation.

Novel 3-dimensional analyses of blind deconvolved images of the fat system studied were successful in demonstrating several previously theorized characteristics of fractal

scaling properties. This included experimental evidence for diffusion-limited cluster aggregation, and evidence for fractal dimension retention upon collapsing of an object in 3D onto a 2D plane. Furthermore, results of a novel quantitative measure of order, along with measurement of area fraction, provided evidence that supports the belief that the fractal dimensions D_f and D_b are influenced by both the degree of fill and the degree of order of particle distribution in space. However, the relative contributions of fill and order to fractal dimension values still remains to be conclusively determined.

Comparison of network features in a volume to those in thin films was successful in demonstrating dramatic effects of confinement in thin film growth. This included an increase in area fraction, and the fractal dimension D_f , suggesting that confinement greatly increases the degree of order and fill in thin films, relative to growth in a volume.

For the system studied, many significant correlations were found for a number of network properties. These relationships suggest that growth of large clusters in a volume is 3D in nature, with a large proportion of mass found near the center of the sample. Relationships between these characteristics suggest that the areas with more mass consisted of a higher number of larger particles that displayed higher fractal dimensions.

3-Dimensional volume reconstructions of blind deconvolved polarized light images provided a new means of visualizing fat crystal networks grown in volume conditions. Qualitative assessment of the size of 3D clusters and the relative locations of clusters in 3D space could be made from these renderings, thereby offering a never-before-seen perspective.

Lastly, the results for fractal dimensions D_f and D_b obtained for thin films were shown to be equivalent to those determined under varying experimental conditions including the use of different microscope, camera types, magnifications, and image spatial resolutions.

6. RECOMMENDATIONS FOR FUTURE RESEARCH

The preliminary work in this study suggests that wide-field blind deconvolution of fat systems viewed under polarized light is feasible. However, a number of recommendations for further research are suggested.

6.1. *Verification of the Z-stage Calibration*

The z-stage controller used in this study was assumed to be accurate at the time of purchase and drift from its calibrated state was assumed to be negligible. Since 3D polarized light microscopy is not traditionally performed, obtaining a calibration specimen is difficult if not impossible. There are other methods, however, to assess the accuracy of the z-stage motor. One such method was proposed by Barry Burbach and shared by Martin Wessendorf (Wessendorf, 2003) and involves the use of spherical glass beads around 10 μ m to 30 μ m in diameter. Beads are mounted in the mounting medium that is doped with a fluorescent dye. Z-series are acquired and their XZ profiles acquired. If the microscope is correctly calibrated in XY and assuming that the beads spherical, the z-calibration can be deduced from the average of the ratio of the bead diameter in the x-direction to that in the z-direction. If the z-stage is properly calibrated the cross-section of a sphere should appear to be a circle. By verifying the z-stage calibration, the cause of the appearance of elongated crystals in the XZ profile could be better assessed (3.1.4). Alternatively, the use of a bead coated with fluorescent dye may be used (McNally et al., 1998).

6.2. Various Systems, Crystallization Conditions, and Degrees of Confinement

Since this study was conducted on one model fat system, under specific crystallization conditions, it is highly recommended that future efforts aim to validate the findings within, and determine the limitations discussed in section 4.2. By repeating this study with various fat systems, under similar conditions, support for the findings within and benchmarking of limitations would be of great value. In addition, studies employing the same model fat system with altered crystallization conditions may reveal differences due to crystallization conditions. It is well known that the crystallization of fats is highly influenced by factors including the rate of cooling, the degree of supercooling, and the presence of shear (refs) and learning more about the effects of these parameters on the crystallization of fats in volumes would be advantageous. Furthermore, by altering the thickness of the volume sample, studies may reveal differences in network formation resulting from various degrees of confinement.

6.3. Other Deconvolution Algorithms

Although blind deconvolution has obvious great benefits over methods that require knowledge of the point spread function, and although it has been demonstrated as a successful means of imaging 3D volumes, investigations of other algorithms may be of value. Such other methods may include classical iterative algorithms (Agard's modification of the Jansson-Van Cittert algorithm for example), and statistical iterative algorithms (the Maximum Likelihood Estimation, for example) (Wallace et al., 2001). While these methods

require a measured PSF, they may or may not produce better deconvolution results. The only way to be certain would be to try them, although obtaining an accurate PSF will admittedly be difficult.

6.4. 4-Dimensional Imaging

This study was successful in investigating network characteristics in 3-dimensions (X, Y, and Z). By including time as a fourth variable, it may be possible to perform multiple z-series acquisitions thereby allowing us to observe the growth of 3D networks as a function of time (Mohler & White, 1998; Thomas & White, 1998; Kriete, 2000). This would be of great value and would address many unanswered questions related to the growth of network features. For example, the locations of early crystal growth, the nature of secondary growth, and the evolution and changes that occur in crystal morphology would be observed. These findings would be of great importance in furthering our understanding of the nature and mechanisms of crystallization and network formation in bulk fat systems.

6.5. Development of a 3-Dimensional Imaging-based Fractal Dimension Method

The development of a 3D imaging-based fractal dimension determination would be far from trivial and would include a number of assumptions. The following is one conceivable approach that may be appropriate for pursuit in the future.

If the 3D volume imaging technique reaches a level of stereological accuracy that allows for high confidence in the ability to quantitatively determine the exact locations of

individual particles in 3D space, an iterative particle-counting procedure could be pursued. Particles embedded in iteratively reduced cube sizes could be counted using an adjusted count that includes features touching 3 of the 6 cube faces. Applying these counts to the fractal scaling model, a log-log plot of the number of particles as a function of cube length may result in a 3D fractal dimension. This would admittedly be far from trivial, but in theory, it could be performed. This analysis would be particularly interesting when compared to the existing model for the determination of 3D fractal dimensions by rheological methods.

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8. APPENDICES

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