III. THE MICROGRAPH

III.A. INTRODUCTION TO IMAGE ANALYSIS & PROCESSING OF BIOLOGICAL SPECIMENS

The terms **image analysis** and **image processing** are often incorrectly assumed to be synonymous, but they refer to different aspects of the treatment of image data. **Image analysis** involves the quantification and classification of images and objects of interest within images. **Image processing** refers to any technique which alters and displays, in more tangible form, the information contained in images**.** An image might be analyzed either before or after it has been processed. Most of this class is devoted to learning about the methods by which electron micrograph images can be processed to obtain clearer images of objects photographed at high magnifications.

Reversal of image contrast when a positive print is made from a photographic negative represents, perhaps, the simplest and most practiced form of image processing. More sophisticated techniques were developed years ago to enhance images of the moon and other celestial objects (*e.g.* Nathan, 1970). Markham, *et al.* (1963) and Klug and Berger (1964) were the first to develop processing techniques to study the structures of biological specimens imaged by electron microscopy. They succeeded in separating structural details relating to the regular features of periodic specimens (**SIGNAL**), from non-regular details (**NOISE**). A primary goal of biological, electron micrograph image processing is to extract accurate structural information from specimen images that are obscured with noise**.**

Image processing extends the electron microscopist's ability to study imaged biological structure because details that may be invisible to the naked eye can be clearly revealed. An obvious benefit of the clearer images and structural information is an enhanced understanding of biological structure-function relationships. Correlation with x-ray diffraction, biochemical, genetic, immunological, and model building studies makes image processing a powerful tool for investigating the basis of molecular events in living systems.

III.A.1. Image Processing Techniques: Real and Reciprocal Space Methods

Since 1963 several techniques have been developed and are now routinely used in biological structure studies. Optical and computer diffraction and filtering, three-dimensional (3D) reconstruction, rotational and translational optical superposition, real space iteration and back-projection, correlation and convolution averaging, and holographic methods are among many that are available. Obviously, certain techniques are better suited than others for studying some specimens. Image processing techniques are classified as either **real-space** (direct) or **reciprocal-space** (Fourier) depending on whether the micrograph or electron image itself is processed or an intermediate step involving the Fourier transformation of the micrograph or image is required for processing. The main emphasis in this course concerns the Fourier analysis and processing of periodic specimens because Fourier techniques are well-established and have been used extensively to study a wide variety of periodic biological systems. As time permits, other, popular techniques will be introduced.

III.A.2. Fourier Image Processing

Fourier image processing, based on the well established principles of X-ray diffraction and X-ray crystallography, was primarily developed in the 1960's and 1970's by A. Klug and colleagues at the Medical Research Council Laboratory in Cambridge, England, and has been the technique of choice in a majority of structural studies of biological macromolecular assemblies. Fourier techniques are most effective for the study of crystalline or paracrystalline specimens. However, in principle, these techniques can be applied to the study of any non-crystalline specimen. Fortunately, a wide range of biological specimens naturally occur or can be isolated *in vitro* as regular arrays and are amenable to this type analysis. Viruses, muscle proteins, membranes, ribosomes, flagella, microtubules, enzymes, pili, chromatin, etc. have all been successfully examined by Fourier-based image processing techniques.

Fourier processing may be performed **optically** on an optical bench or **digitally** on a computer**.** Both techniques have distinct advantages and disadvantages which are discussed in this course (Sec. III.D.3.b). It should be emphasized, however, that there are other quite powerful and

quantitative techniques such as correlation methods that offer distinct advantages over Fourier techniques for examining certain kinds of specimens (*e.g.* disordered or non-periodic specimens). If time permits, some of these techniques will be discussed.

III.A.3. Periodic/Non-Periodic Specimens

Except where noted, we will concentrate on studies of **ordered specimens** (*i.e.* containing regular arrangement of "subunits"), and **specimens prepared by conventional negative staining or cryo-microscopy techniques** imaged under normal or minimal exposure conditions of bright field transmission electron microscopy (60-100 kV at 10,000-100,000X). Section III.C.4 discusses the fundamentals of Fourier (diffraction) theory and related topics to help facilitate more effective application of Fourier techniques and to identify the capabilities and limitations of the techniques. Literature references provide the rigorous mathematical treatments omitted here in favor of more illustrative descriptions. Inherent assumptions and the effects of sample preparation and imaging are discussed (Sec. III.F) to emphasize ways to improve the quality and significance of results obtained by image processing. Technical details of optical diffraction (Sec. III.D.1) and filtering (Sec. III.D.2) are compared with other analysis methods (Secs. III.D.3.b and III.G). Section III.H discusses the display and interpretation of final results.

III.A.4. Applications and Advantages of Image Analysis/Processing

The following list identifies many of the common uses of image analysis/processing.

a. Objective means to reveal, assess, and measure periodic structural detail

Lattice dimensions (unit cell size and shape) are more accurately measured than by direct means. Dimensions aid in estimating molecular weights and identifying chemical species.

Detection of rotational and translational symmetry elements.

Detection of specimen preservation (distortion, resolution, staining artifacts). This provides a good criteria for selecting suitable images for further analysis. Some types of specimen distortions (*e.g.* crystal lattice distortions) can be reduced or removed with specific analysis techniques.

Determine average positions, size, and shape of subunits.

Identify and separate signal and noise components in images.

Separate Moiré images from multi-layered specimens.

b. Use image averaging to improve contrast, reduce noise, and improve resolution over what could be obtained in a single image. Averaging allows particular specimen features to be enhanced.

c. Recover (reconstruct) 3D information from 2D images.

d. Determine and adjust the imaging conditions and assess the quality and conditions of microscopy (astigmatism, focusing, specimen, and focal drift, and beam coherence).

e. Determine handedness of structures.

f. Measure specimen thickness.

g. Determine or verify chemical stoichiometries.

h. Detect conformational changes in macromolecules.

i. Study macromolecular assembly and the molecular events leading to movement.

j. Perform image restoration operations - (deblurring, contrast stretching, histogram equalization, low and high-pass frequency filtering, etc.).

k. Teaching aid for understanding diffraction theory and methods.