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ANNUAL REPORT

LASER SCATTERING TOMOGRAPHY FOR THE STUDY OF DEFECTS IN PROTEIN CRYSTALS

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INTRODUCTION

The goal of this research is to explore the application of the non-destructive technique of laser scattering tomography (LST) to study the defects in protein crystals and relate them to the x-ray diffraction performance of the crystals. LST has been used successfully for the study of defects in inorganic crystals and, in the case of lysozyme [1], for protein crystals.

Several approaches have been proposed to assess protein crystal quality and to identify the defects in the crystal. X-ray topography has been a successfully applied to the study of defects in both inorganic and organic materials. However, the diffraction contrast in protein crystals is so poor even using synchrotron sources [2] that it appears that this technique will not yield defect information for these crystals. It also shares the common failing of all x-ray techniques that the crystal quality is degraded [2] rendering the crystal unusable for a subsequent structural determination.

Mosaic spread has been used as a measure of protein crystal quality. X-ray goniometry has been employed by a number of investigators [2,3,4] to study mosaic spread in protein crystals. However, this technique does not identify the type or density defects although it is possible to infer the range over which the defects extend [3].

Durbin and Feher [5] have applied transmission electron microscopy (TEM) to the study of protein crystals. The process, particularly sample preparation, is rather complicated and involves freezing the protein crystal and subliming the solution around the crystal. The crystal may then be cleaved to look at interior features. The surface to be studied must be replicated and the replica shadowed for use in the microscope. This approach has revealed the spiral growth steps associated with a screw dislocation in lysozyme. It has not been extended to study the number of these defects generated in the crystal under differing growth conditions. The crystal is also destroyed in the replication process.

Sato et al [1] have studied etching as a means of revealing defects in protein crystals. This approach has revealed both point and line defects in lysozyme. Unfortunately, this approach requires large crystals which are destroyed in the process.

Scanning tunneling microscopy (STM) and atomic force microscopy (AFM) have been applied to the study of protein crystals [6,7,8]. With proper manipulation of the data, it is possible to get near molecular resolution for protein molecules [8]. Durbin [7] has shown steps and ledges

on the surface of growing lysozyme crystals. Recently, AFM studies have revealed defects at the surface of protein crystals. Since both STM and AFM are surface techniques, they can potentially only give information about defects which intersect the surface. Both techniques do leave the crystal undamaged for structural determination.

Laser scattering tomography is, in principle, a simple technique. A focused, low power laser beam is directed into the crystal and the scattered light is observed at 90° with a microscope. The image is recorded either on film or, as has become more common, as a video image. The scattering centers are related to defects and occur due to fluctuations of the dielectric constants at the lattice defects [9]. Two basic types of defects can be identified: point defects [9] and line defects [10,11,12]. The point defects are typically much smaller than the wavelength of the analyzing light and can be treated a classical Rayleigh scatterers [9]. The analysis of the line defects is more complicated [9,11,12] and is hampered for proteins by the lack of data for the physical parameters such as the dielectric constant [1]. However, as the work of Sato et al [7] has demonstrated, this does not materially compromise the use of LST to study defects in protein crystals. The basic types of defects can be classified by their interaction with the polarization of the analyzing light .

Laser light scattering tomography can be compared to x-ray topography (XRT) and there are some definite advantages to the use of LST. The most obvious advantage is that the protein crystals are not degraded by LST. The in-plane resolution of both LST and XRT are the same; about 1μ [9]. The resolution out of plane is limited by the diameter of the light beam for LST (≈ 20μ) while that for XRT is limited by the specimen thickness of approximately 1mm [9] which will yield better defect resolution for an LST scan. There is a close association between the laser scattering tomographs and the x-ray topographs, since they both depend on the shift of electron densities caused by defects in the crystal. The differences arise because the light is scattered from the outer bonding electrons while the x-rays are scattered from the core electrons [11]. A major advantage of LST comes from the greater transmittance of the optical beam compared to x-rays. This allows the full depth of the crystal to be probed and allows the study of defects related to the growth history of the crystal [11]. This type of data will be used to further the understanding of the growth process in protein crystals.

The overall goal of this program can be subdivided into 5 intermediate goals: 1) build a laser scattering tomography apparatus, 2) study the defects in protein crystals, 3) study the effects of growth conditions (solution concentrations, precipitants, impurities, temperature and gravity) on these defects, 4) correlate defect type and density in the crystal with the quality of the x-ray

diffraction from the crystal, and 5) develop a predictive relationship between the LST images and the quality of the crystal for diffraction studies.

The goals for the first year of the program were to build and test the LST apparatus (9 months) and to begin the comparison of terrestrial and space grown crystals studying both the defects and the x-ray diffraction data.

PROGRESS TO DATE

Apparatus

The nominal starting date for this program was July 1, 1995. However, funding was not available until September 1995 and complete staffing of the program was delayed due to medical reasons. The effect of both of these occurrences was to delay the actual start of the research for three months.

The design of the LST apparatus involved the development of two major systems: 1) the hardware system which performs the light gathering and image storage functions, and 2) the control program which integrates the electronic functions of the apparatus and manipulates the images. The basic hardware system of the LST apparatus is shown schematically in figure 1. This system can be further broken down into five sub-systems: the illumination system, the crystal positioning system, the imaging system, the image processing system and the computer. The illumination sub-system consists of a laser light source and the focusing optics which produce a 20 to 40μ diameter spot with a long depth of focus providing uniform illumination across the width of the crystal.

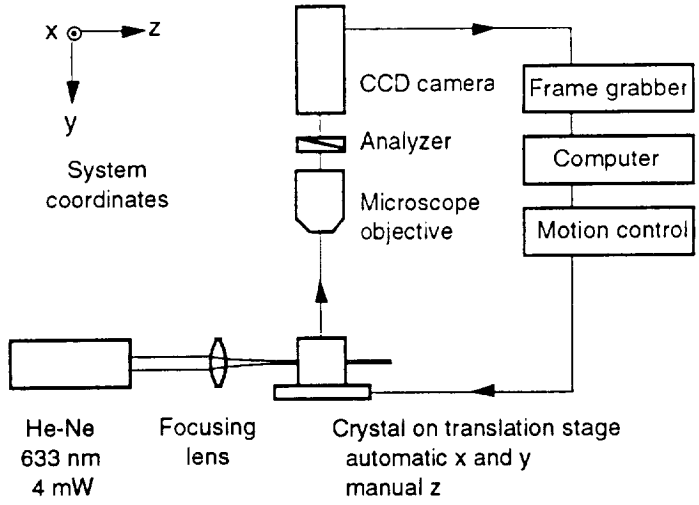


Figure 1. Schematic diagram of Laser Scattering Tomography (LST) apparatus

A motorized XYZ stage and the associated motor control electronics is the crystal positioning sub-system. This sub-system provides the movement necessary to build up the image of the defects in the crystal. This image is built up by striping out the part of the image that corresponds to the illuminated area of the crystal and transferring it to an image buffer. Then the stage is moved in the x-direction and the next segment of the image is appended to the buffer adjacent to the previous one. By repeating this process across the length of the crystal, an image of the defects in a thin (20 to 40μ) slice of the crystal is obtained. The whole volume of the crystal can be studied by moving the crystal in the y-direction and repeating the scan. Thus, it is possible to study the spatial distribution of the defects in the crystal (see for example Sato et al. [1]).

The imaging sub-system consists of a microscope and a CCD camera. The microscope must provide a range of magnifications such that the crystals can be imaged in their entirety in one frame. The objective lenses must also provide sufficient working distances to allow the crystal to be imaged to its full depth when contained in the cell which maintains its hydration. An analyzing polarizer in the microscope is necessary to aid in the identification of the types of defects in the crystal (Moriya and Ogawa [2,3,4] and Moriya[5]). The CCD camera must have sufficient sensitivity to detect the scattered light from the crystal.

The image processing sub-system (frame grabber) provides the electronic means of storing the image from the CCD camera. It also contains the buffers used in building up the final image of the crystal and performs some of the functions required to process that image.

The final sub-system is the computer. The computer will oversee the functions of both the positioning and the image processing sub-systems. It will provide the timing to ensure that image acquisition and processing, and motion are not attempted at the same time. The computer will also perform some of the operations necessary for the image generation.

The main task in the design of the apparatus was the selection of the components of the system. Of particular importance, was the selection of the video board and the motion control system since these must be compatible within the computer environment used for controlling the system. Because of the number of imaging and motion control systems supported by PC type computers, a PC compatible computer was selected for the system. Watcom C/C++ was chosen as the programming language since it is compatible with possible expansion to the National Instruments Labview/Labwindows operating system. A National Aperture motion control system and an EPIX 4MEG VIDEO Model 10 video board were chosen from a number of options since they would operate in the desired computer environment. An Olympus BX30M microscope with 2x, 5x, 10x and 20x interchangeable, long working distance objectives and fitted with a Sony CCD camera is used to focus and image the scattered light.

The completed apparatus is shown in figure 2. The laser source for the instrument is a red (632.8nm) 3mw HeNe laser polarized 500 to 1. (This laser may be changed to a green or blue one

to take advantage of the $1/\lambda^4$ dependence of the scattering cross section and the increased resolution possible at the shorter wavelength.) The laser is mounted so that the beam can be traversed in the x- (front to rear of optical table) and y- directions and the beam angle can be adjusted in both the x-y and y-z planes to facilitate alignment with the microscope optics. The mount also allows the laser to be rotated to change the direction of the polarization. The laser beam is focused by a microscope objective lens mounted on an XYZ stage with tilt capability to ensure that the focused beam is aligned with the original path of the laser beam. The crystal under study is mounted on the National Aperture XYZ stage below the microscope. The x- and y-directions of the stage are under the control of the computer; the y-direction is manually adjustable to position the crystal under the microscope optics. The maximum movement on any axis is 0.5in. The crystal mounting area of the XYZ stage incorporates back lighting using fiber optic technology to allow normal viewing and photography of the crystal for comparison with the tomographs. All of the sub-systems except the computer and its peripherals are mounted on a small optical table which is isolated from the bench top to minimize problems with vibration. Also visible in figure 2 (from the left) are the video monitors which can display either the entire frame from the camera or the composite image generated by the computer and video board, the Sony power supply/camera adapter, the National Aperture motor control amplifiers, and the computer system. The hardware was assembled to the point shown in figure 2 by mid-April 1996 and was capable of producing images of scattering from defects (though not full tomographs). Figure 3a and b show images that were obtained from a calcium molybdate crystal. These tomographs clearly show the effect of the polarization of the incident and scattered light on the images.

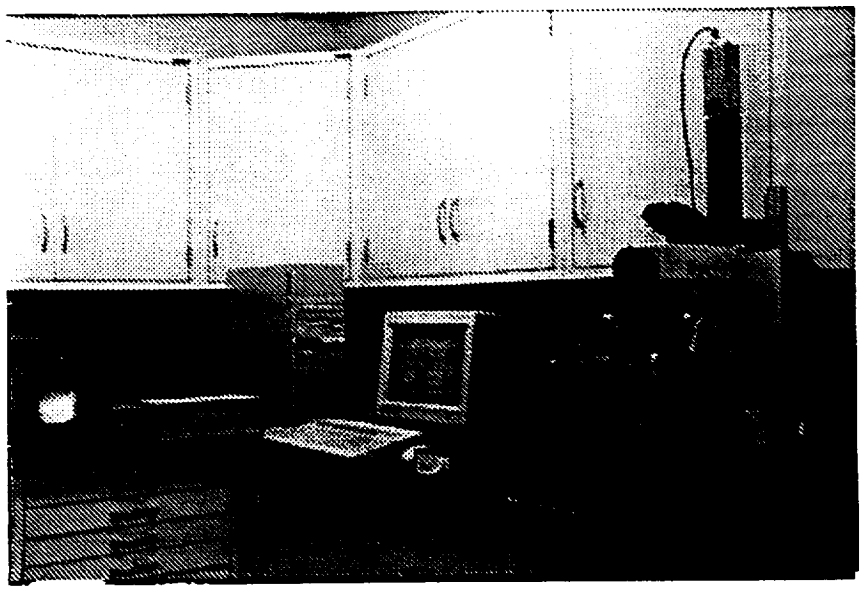
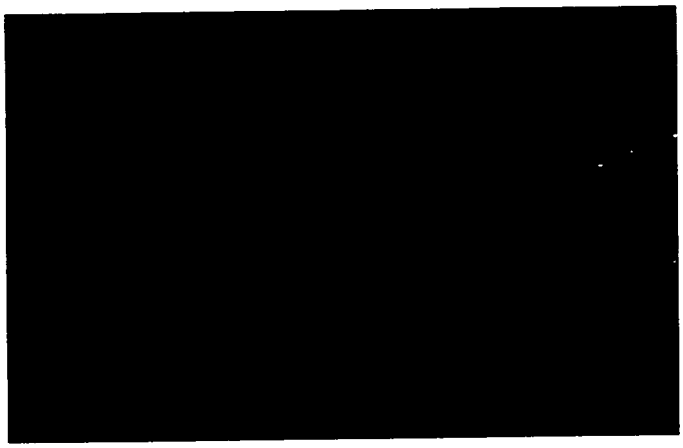


Figure 2. Photograph of LST apparatus at the Center for Materials Research.

I)



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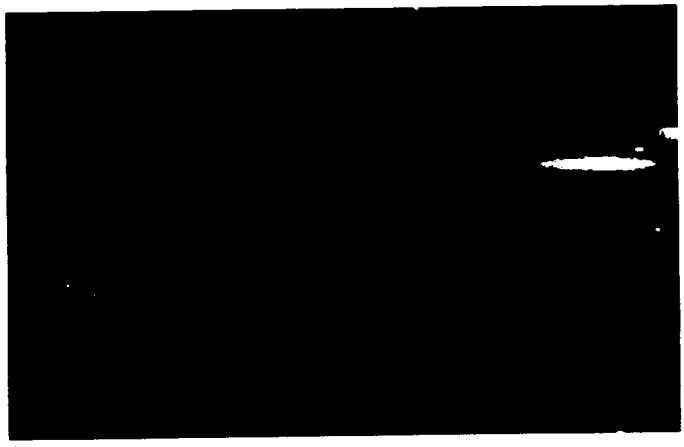
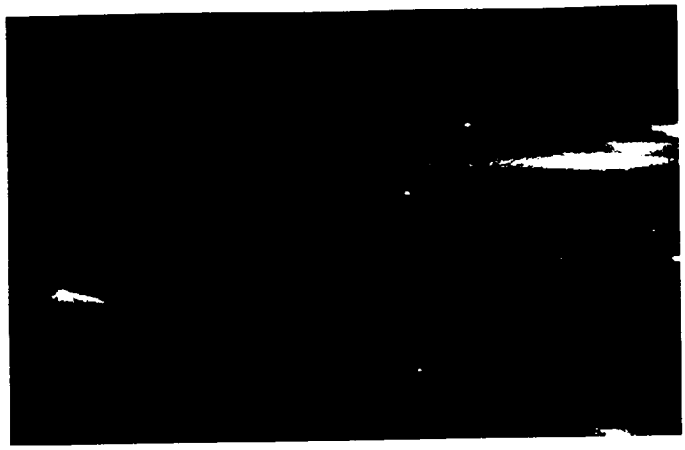


III)

Figure 3a. Tomograph of a calcium molybdate crystal. Laser beam enters from the left and is polarized vertically to the plan of the paper. I) no polarizer in the beam. II) polarizer oriented perpendicular to the direction of the incident beam and III) polarizer oriented parallel to the direction of the incident beam.



I)

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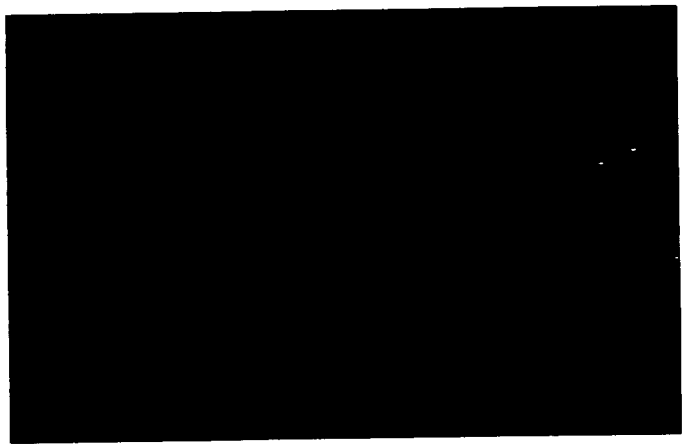

III)

Figure 3b. Tomographs of a calcium molybdate crystal. Laser beam enters from the left and is polarized in the plane of the paper. I) no polarizer in the scattered beam. II) polarizer oriented perpendicular to the direction of the incident beam and III) polarizer oriented parallel to the direction of the incident beam.

Control Program

Concurrently with the final assembly of the hardware, the control program which integrates the motion control, and video acquisition and manipulation was being developed. This program must perform a number of functions in order to control the system including:

1) Record initial starting position in the x- and y-directions
2) Index initial x starting position to the limit switch
3) Interrogate the operator for the magnification, laser beam size, crystal size in the x-and y-directions, step size in the y-direction and the file name
4) Move crystal to initial position in x
5) Place full frame image in image buffer
6) Strip out image pixels corresponding to laser path
7) Place pixels in data buffer
8) Step crystal in the x-direction
9) Place new full frame image in image buffer
10) Strip out pixels corresponding to laser path
11) Place pixels in data buffer adjacent to previously collected data
12) Repeat move and data collection until full image is collected
13) Store image of layer
14) Return to initial position in x and step in the y-direction
15) Repeat data collection for next layer
16) Repeat until data is collected through the full y-distance
17) Return crystal to initial x and y positions
18) Notify operator that scans are complete

This program which is written in C/C++ and utilizes the library functions provided by the manufacturers of the image and motion control boards is 1200 lines of code long and is currently being debugged. When this task is completed, the Laser Scattering Tomograph will be fully operational and the study of the defects in protein crystals will begin.

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