Optical Monitoring of Protein Crystal Growth

Final Report
Submitted to NASA/MSFC under Grant # NAG8-599

by
A. Choudry
Center for Applied Optics
University of Alabama in Huntsville
May 30, 1988
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I. Introduction

Determination of the three dimensional structure of the protein crystals is crucial in understanding their biochemical properties. In particular, this is of considerable interest to the pharmaceutical and biotechnological industries. Knowledge of the three dimensional structure of a protein can be used to design compounds that selectively bind to protein sites and thereby inhibit or otherwise modify the activities of the protein. Currently, X-ray crystallography is the only well established technique to determine three dimensional structure of complicated biological macromolecules such as proteins. Furthermore, it is believed that no other technique will be competitive with X-ray crystallography in the near future for routinely determining three dimensional structure of large biological molecules.

It is relatively easy to grow small (~100 μ) protein crystals, however, for a successful determination of the three dimensional structure, X-ray crystallography requires rather large size (a few mm) crystals. Despite the development of a variety of crystallization methods, it remains difficult, in most cases, to either nucleate protein crystals or grow them large enough and of high enough quality for X-ray studies. It is believed that gravitational convection currents play a strong inhibitory role in the crystal growth. A space platform, with the absence of gravitational convection currents, should provide a superior environment for protein crystal growth. The major motivation behind the study of crystal growth on space platforms, is the expectation that the elimination of density-dependent flow effects, which occur during crystal growth on earth, may beneficially alter the crystal growth process (1,2,3). The absence of buoyant forces on the crystal also eliminates the problem of sedimentation and thereby keeps the growing crystal in a uniform solution. Contact with vessel walls can also lead to heterogeneous nucleation, however, in the absence of gravity, it is possible to perform containerless crystal growth where stable spherical droplets of solution can be suspended by acoustical or electrostatic levitation.

For successful crystallization it may become important to adjust growth parameters such as pH, vapor pressure etc., just after nucleation. Detection of nucleation is thus an important step. Only non-invasive techniques for the detection of nucleation can be considered and optics is an excellent candidate for it. Under this project we have investigated the possibility of using various optical techniques and interferometry in particular for detecting the onset of nucleation in protein crystal growth.
2. **Optical Techniques**

Optical techniques have been widely used in studying crystal growth. Protein solutions consist of four components, namely, water, a buffer, protein and a precipitant and the protein solubility and its concentration can be varied independently by the precipitant (4). These solutions are in general, clear and transparent with an index of refraction close to that of water. On set of nucleation, in principle, should manifest itself as local fluctuations in the refractive index. Optical techniques have been extensively used for studying crystal growth, including in space environment (5 - 12). For our optical experiments we chose lysozyme crystal growth since its chemical nature is being studied elsewhere in the NASA Program [13, 14]. Although there is an extensive literature on crystal growth and optical techniques, however, we have not been able to trace references to the use of these techniques for the crystal growth of lysozymes in the library search as shown in Appendix A.

There are several optical techniques available to detect these fluctuations in the local refractive index:

These include:

- Direct microscopy
- Scattering
- Interferometric microscopy
- Ultraviolet absorption
- Polarization
- pH Value

2.1 **pH Value**

The latter being largely non-optical is still included in the list since the pH value seems to be a critical parameter in protein crystal growth. In a future study, the possibility of using pH value should be systematically investigated. There are, however, attempts to use Fiber-Optic sensors for the measurement of pH [16]. These are based on injecting a minute amount of dye into the solution. This is too drastic a perturbation for the protein crystals and is thus unacceptable. In any case, pH-sensors are not able to determine the hydrogen ion concentration with any spatial resolution but only give an average value over a certain volume.
2.2 Direct Microscopy & Polarization

Direct microscopy has only limited value except for seeing the crystals after they have reached a certain size, typically 10 microns. Furthermore, generally microscopy gives only qualitative (non-metric) information and as such not useful for quantitative control of the process. Direct microscopy in combination with polarization, say in the form of Nomarski microscope, may be a valuable tool since almost all bio-chemicals exhibit some optical activity. This point was considered in this project and we did try to modify a commercial microscope to combine with our interferometric setup (described later). The preliminary results were encouraging, however, a much more sophisticated setup either in the form of commercial Nomarski microscope or substantially modified setup is needed. This could not be pursued under the limited scope of the present project. This is a very promising approach and should be included in future studies.

2.3 General Metrologic techniques

The general problem in all optical metrologic techniques, be it interferometry or scattering or any other variant, is that a priori knowledge of the geometry of the solution (drop, cuvette, prism between slides etc.) is required to find the optical parameters (refractive index, optical activity etc.). Measurement of such optical parameters for an arbitrarily shaped object, e.g. an oscillating hanging drop defies all the common techniques. All the other efforts under NASA that we know, and as reported in various meetings are based on the so called 'good-geometry' configuration which is not quite the case for the eventual experiment with 'hanging-drops'. We studied this problem at some length and found that more sophisticated techniques based on Radon Transforms or Tomography could be helpful. A discussion of these points and allied subjects was presented at a presentation at UAB on Dec. 19, 86 and is appended as Appendix C A request to acquire appropriate software to carry out such investigations was made but it was denied by the contracting authorities.
2.4 Light Scattering

Light scattering is a very powerful technique to detect local fluctuations in the refractive index if the geometry of the scatterer remains well defined during the observation. At its best, Light scattering gives a statistical average of the state of nucleation and could thus furnish an average indication of the onset of nucleation. It certainly cannot answer such questions as the density profile around a crystal. In any event a detailed study of light scattering and its relation to the extent of nucleation has yet to be carried out and is strongly recommended. We did perform a rudimentary study of the theoretical background as described in Appendix B.

2.5 Ultraviolet Absorption

Ultraviolet absorption, particularly in the region of 280 nm, is, again, a very powerful technique as it relates to protein concentration (17). It has been one of the traditional techniques used in protein crystal growth [13]. Again it will give only an average indication of the nucleation. Kam et. al. [15] obtained UV light transmission photographs of growing lysozyme crystals using a light source with a wavelength of 277 nm. This technique allowed them to show the depleted area around the growing crystals. But it is not sensitive enough to detect a concentration change due to the presence of aggregates or very small crystals in the solution. A quantitative study, suitable for real-time closed loop control of the protein crystal growth, however, seems to be lacking and should be investigated eventually. We tried to fiber optically couple a dual wavelength UV source to the Petri-dish setup. This was in direct conflict with the coated surface required by the interferometer. We did, however, succeed in putting the fiber in a different region of the solution and observed UV absorption during crystallization. Except for a broad agreement with already well known facts we could not get conclusive results from this setup. A detailed study would require a serious modification of the interferometric setup and was thus not further pursued.
2.6 Interferometry

Interferometry, as direct interferometry or in various guises, e.g., microscopic, schlieren, diffraction, speckle, holgraphy, etc., has been extensively investigated and used in crystal growth studies. We used two different types of microscopic interferometers. The first one was based on a Petri-dish with 'coated' surface and an integral microscopic interferometer, as shown in Figs. 1--4. A drop of the protein solution placed on the petri dish was used in this study.

The lysozyme was dissolved in distilled water and then extensively dialyzed against pH 4.0 buffers. The buffered lysozyme solutions were sterilized by passage through a Nalgene 0.2 micron filter and then concentrated in a sterile Amicon Ultrafiltration cell. Solutions of 5% NaCl and 30 mg/ml were also prepared in these buffers and sterilized. At room temperature (20°C), the solubility of the solutions was 6 mg/ml [13].

Details of the optical setup we developed and used in the interferometric study along with the results obtained, were presented at the J. Wheeler State Lodge meeting on May 14, 87 and is appended as Appendix D. In Fig. 5 a typical interferogram with a possible perturbation due to nucleation (lower left corner) is shown. In this setup we have observed diffraction rings possibly also due to nucleation as shown in Fig. 6.

Next, we used a commercial Interferometric Microscope (Leitz Metalloplan; courtesy of the Center for Microgravity and Materials Research, UAH) to both directly view and examine interference patterns during the crystallization of Lysozyme protein. Details of this optical setup and the results so obtained were presented at a meeting on Nov. 4, 87 at MSFC (along with a video tape), Appendix E and at an International meeting at Cannes, Appendix F.
Fig. 1 and 2. Microscope Interferometer
Fig. 3 and 4. Microscope Interferometer
Fig. 5 Interference pattern with possible nucleation site

Fig. 6 Diffraction pattern with possible nucleation site
3. Conclusions

For optical monitoring of protein crystallization it is essential to first collect basic data on the optical properties of the solution. A lack of this data in the available literature, severely hampered our design and analysis of the experiments. Before attempting to seriously devise space worthy monitoring techniques we believe that separate 'good-geometry' experiments should be carried out just to measure at least the following optical properties:

-- refractive index
-- dispersion
-- UV absorption
-- optical activity
-- scattering

of the protein solution under varying conditions. Furthermore, it would be of immense value if some such measurements could also be carried out protein crystals themselves.

Without a base of such fundamental data it is not possible to make scientifically sound evaluation of monitoring techniques.
References

[3] L.J. Delucas and al. - Preliminary investigations of protein crystal growth using the space shuttle
[6] J C Van Dam and al. - The application of a new simple interference technique to the determination of growth concentration gradients of the layer perovskite NH$_3$(CH$_2$)$_3$NH$_3$CdCl$_4$
J. of Crystal Growth 84 (1987) 539-551
Appendix A

Library Search

File 13:INSPEC - 77-87/15822
(COPR. IEE 1987)

S1 15 PROTEIN()CRYSTAL? AND LYSOZYME?
S2 8 S1 AND GROWTH

? s s1 and refractive()index
S3 0 S1 AND REFRACTIVE()INDEX

? s s1 and optical()activity
S4 0 S1 AND OPTICAL()ACTIVITY

? s s1 and interferometry and growth
S5 0 S1 AND INTERFEROMETRY AND GROWTH

? s s1 and ultraviolet()absorption and growth
S6 0 S1 AND ULTRAVIOLET()ABSORPTION AND GROWTH

? s s1 and polarization
S7 0 S1 AND POLARIZATION

? t s2/7/all
Appendix B

Light-scattering

In a light-scattering experiment, a laser beam impinges on the scattering medium. The scattered light is analyzed by a detector, whose position defines the scattering angle \( q \) (Fig. 1B).

Fig. 1B a schematic representation of the light-scattering experiment.
In modern experiments, the time-correlation function of either the scattered field or the scattered intensity (or their spectral densities) are measured. The detector is composed of a photomultiplier followed by an autocorrelator or a spectrum analyzer. The three different methods used, called filter, homodyne and heterodyne methods are schematically illustrated in fig 2B.

![Diagram](image)

Fig. 2B: Schematic illustration of the various techniques used in light-scattering experiments. (a) filter methods. (b) homodyne. (c) heterodyne.

It is typical of solutions of biological macromolecules that:
- the polarization of a macromolecule is enormous by comparison to the polarization of a solvent molecule.
- macromolecules move much more slowly than solvent molecules.

Macromolecules are thus more efficient scatterers and macromolecular motions are theoretically temporally separable from the solvent motion. Macromolecules dominate the long-time behavior of the solution and are the only molecules to be taken into account when studying the light scattered by the solution. Accordingly in a protein solution, we have to take into account the light scattered only by the protein molecules or by the agregats of protein molecules.
**Time correlation functions and spectral densities**

Let $E(q,t)$ be the electric field which is detected by the detector. The time-correlation function and the spectral density are defined by:

$$F(q,t) = \langle E^*(q,0)E(q,t) \rangle = \lim_{T \to \infty} \frac{1}{T} \int_{-T/2}^{T/2} E^*(q,t+t)E(q,t)dt$$

$$F(q,w) = \text{FT} [F(q,t)] = \int_{-\infty}^{\infty} F(q,t)e^{-iwtdt}$$

If the scattering molecules are spherical then the heterodyne correlation function and its corresponding spectrum are:

$$F_1(q,t) = \langle N \rangle \exp^{-q^2D|t|}$$

$$F_1(q,w) = \rho^{-1}\langle N \rangle q^2D / (w^2 + (q^2D)^2)$$

where $q$ is the wave number and $\langle N \rangle$ the average number of molecules in the scattering volume.

The homodyne correlation function and its associated spectrum are given by:

$$F_2(q,t) = \langle N \rangle^2 + \langle N \rangle^2 \exp^{-2q^2D|t|}$$

$$F_2(q,w) = d(w) \langle N \rangle^2 + \rho^{-1}\langle N \rangle (2q^2D) / (w^2 + (2q^2D)^2)$$

Heterodyne or homodyne light-scattering can be used to measure the diffusion coefficient of a macromolecule by determining either the time correlation function or the spectral density. The radius $a$ of the molecule and the diffusion coefficient $D$ are bound by the relation:

$$D = k_BT/6pha$$

where $h$ is the viscosity of the solvent, $T$ is the temperature and $k_B$ is the Boltzmann constant.
Light scattering experiments can thus provide information about the size of the macromolecules in the solution. In a protein solution, \( j \) protein molecules can gather to form a bigger molecule (\( j \)-mer). If we note \( <N_j> \) the average number of \( j \)-mers in the scattering volume, the time-correlation functions and their associated spectra will be given by:

\[
F_1(q,t) = \sum <N_j> \exp(-D_j q^2 |t|)
\]

\[
F_1(q,w) = \sum p^{-1} <N_j> \left[ q^2 \left( \frac{1}{w^2 + (D_j q^2)^2} \right) \right]
\]

\[
F_2(q,t) = \sum \left[ <N_j>^2 + <N_j>^2 \left[ \exp(-2D_j q^2 |t|) \right] \right]
\]

\[
F_2(q,w) = \sum <N_j>^2 \left[ d(w) + p^{-1} \frac{2D_j q^2}{w^2 + (2D_j q^2)^2} \right]
\]

If \( i < j \) then \( D_i > D_j \). For a solution consisting only of \( j \)-mers, the half-width at half maximum is given by:

\[
D_{w_j} = D_j q^2
\]

The formation of \( j \)-mers in the solution will lead to a narrower spectrum. Thus it should be possible to follow the formation of aggregates when determining the spectrum of the light scattered by the solution.
Appendix c
Presentation at UAB 12/19/86

Optical Monitoring of Protein X-Tal Growth

A. Choudry

Center for Applied Optics

UAH
PROTEIN SOLUTION

1 mm

[DETECTOR]
PHOTOMETRIC

SIGNATURE

PATTERN RECOGNITION
CORRELATIONS
BLUE,
MODEL BASED
VISION
PROJECT AIM

- IMAGING

- UV ABSORPTION

- REFRACTIVE INDEX
- IMAGING

- ILLUMINATION (1)

- ACQUISITION (2)

(1) - DIFFUSED ILLUMINATION

- COLLIMATED ILLUMINATION

(2) - SINGLE VIEW

- MULTIPLE VIEW COMPOSITE
In principle one gets an albedo map
(or surface reflectance map).
It may not have a strong A-dependance.
UV-ABSN: &

Refractive Index Are

Generically Related To

The Complex R.I.

\[ n = a + ib \]

\[ n(\vec{r}) = A(\vec{r}) + ib(\vec{r}) \]
\[ I(\gamma) = I_0 e^{-(a(\gamma) + ib(\gamma))} \]

- **Phase Modulation** \( b(\gamma) \)
- **Amplitude Modulation** \( a(\gamma) \)

**Coupled with These Are**

**The Geometrical Boundary Effects**
PHASE MODULATION

INTERFEROMETRY

RADON TRANSFORMS (TOMOGRAPHY)

PHOTOMETRIC SIGNATURE

ALSO FOR AMPLITUDE MODULATION
INTERFEROMETRY

ONLY RELATIVE PHASE
& STRONGLY DEPENDENT ON GEOMETRY

HOLOGRAPHIC - INTERFEROMETRY
[REAL-TIME & DOUBLE EXPOSURE]
OPTICAL MONITORING
OF
PROTEIN CRYSTAL GROWTH

A. CHOUDRY
CENTER FOR APPLIED OPTICS
U A H, HUNTSVILLE AL
GOAL

1-3 mm

LYSOZYME SOLUTION DRC

-- IMAGING OF THE DROP

-- OBSERVE THE ONSET OF NUCLEATION DURING CRYSTALISATION

-- DETERMINE SIZE OF NUCLEATION SITES

-- DETERMINE pH VALUE
IMAGING

TWO PRIMARY COMPONENTS

-- SOURCE OF ILLUMINATION &

-- IMAGING OPTICS

ORDINARY MICROSCOPE IS TOO BULKY & LIMITS ACCESS TO THE DROP FOR OTHER OPERATIONS

FIBER OPTICS & MICRO-OPTICS A POSSIBLE CHOICE

A TRADE-OFF BETWEEN MAGNIFICATION, DEPTH OF FOCUS & FIELD OF VIEW

ALSO CONFRONT THE PROBLEM OF IMAGING A CURVED SURFACE WHICH IN ITSELF ACTS AS AN OPTICAL ELEMENT
TO OBSERVE NUCLEATION SITES
THERE ARE THREE GENERIC
techniques

1. DIRECT MICROSCOPY

2. SCATTERING

3. INTERFEROMETRY

Direct microscopy is of not much value (discussed earlier)

Scattering is a practical alternative with some limitations
SCATTERING

SCATTERING SITES ARE ANOMALIES OR DISCONTINUITIES IN THE COMPLEX REFRACTIVE INDEX OF THE SOLUTION

A STATISTICAL ESTIMATION OF THE SIZE OF SCATTERING SITES CAN BE OBTAINED

NO LOCALISATION OR VIEWING OF NUCLEATION SITES POSSIBLE

OTHER PROBLEM WITH SCATTERING IS THE PRECISE ALIGNMENT OF THE SCATTERING BEAM IN THE DROP
MICROSCOPIC INTERFEROMETRY

THE FIELD OF VIEW IS ABOUT 200-500\(\mu\) AND FEATURES (RELATED TO REFRACTIVE INDEX VARIATIONS) CAN BE RESOLVED IN THE RANGE OF 10-30\(\mu\)
FOR ANY OPTICAL TECHNIQUE e. g. SCATTERING OR INTERFEROMETRY, IT IS IMPORTANT TO MEASURE THE COMPLEX REFRACTIVE INDEX OF THE PROTEIN SOLUTION AS A FUNCTION OF BOTH WAVELENGTH AND CONCENTRATION (OR STATE OF CRYSTALLIZATION)

A PRELIMINARY STUDY OF THE REFRACTIVE INDEX VARIATION WITH THE PROTEIN CONCENTRATION HAS REVEALED THE POSSIBILITY OF MAKING THESE MEASUREMENTS WITH THE MICROSCOPIC INTERFEROMETER
OPTICAL MONITORING OF PROTEIN CRYSTAL GROWTH

A. Choudry
Center for Applied Optics
University of Alabama in Huntsville

With special thanks to,
N. Dupuis CAO,
Dr. E. Meehan & Pam Twig, Chem. Dept UAH
The Center for Microgravity Research, U A H
The main Goal is to detect & localise the Nucleation Sites. Possible Methods:

-- DIRECT IMAGING
-- INTERFEROMETRIC MICROSCOPY
-- UV ABSORPTION
DIRECT IMAGING

Factors to be considered

- Magnification
- Resolution
- Field of View
- Depth of Field
- Spectral Dispersion
Basic Parameters

Magnification X50 - 150

Resolution 1 - 3 μ

(200 – 1000 TV lines)

FOV 500 - 1000 μ

Depth of Field 50 - 100 μ

Spectral Dispersion .35 - .65 μ
DIRECT IMAGING and INTERFEROMETRIC MICROSCOPY have been combined into a single optical system.
UV ABSORPTION

Factors to be considered

Source 280 nm
Medium, F-O
Detector
Polarization Effects

Nomarski Microscopy
Appendix F

Presentation at SPIE Conf. at Cannes, Nov. 19, 87

REAL-TIME PROCESSING OF INTERFEROGAMS FOR MONITORING PROTEIN CRYSTAL GROWTH ON THE SPACE STATION

A. Choudry and N. Dupuis
The University of Alabama in Huntsville, Center for Applied Optics
Huntsville, Alabama 35899, USA

ABSTRACT

We have studied the possibility of using microscopic interferometric techniques to monitor the growth of protein crystals on the Space Station. Nucleation in the solution is a critical phase and needs to be monitored carefully. It involves detecting and localizing nucleation sites in their earliest phase with dimensions of the order of 50-100nm. A comparison with the well-known technique of scattering and microscopic interferometry for monitoring the nucleation was made and the latter seems to be much better suited for this monitoring task. In microscopic interferometry, a detailed map of the solution density is made in the form of an interference pattern. From this pattern one should be able to detect the onset of nucleation. This technique offers several advantages, however, for space applications it is essential that the interferograms must be interpreted in an automated real-time manner. We have used techniques of digital image processing to develop a system for the real-time analysis of microscopic interferograms of nucleation sites during protein crystal growth. Some details of the optical setup and the image processing system along with experimental results will be presented.

1. INTRODUCTION

Optical techniques and interferometry in particular, have been used extensively to study crystal growth. These techniques have, however, not been used as widely for studying protein crystal growth. Relatively large (several mm) protein crystals are needed to study their structures by using the standard x-ray diffraction techniques. It is relatively easy to grow microscopic (about 100 micron) protein crystals, however, the growth of large protein crystals is very sensitive to minute perturbations in heat and mass transfer. The controlled, near zero-gravity environment provided by space platforms seems to be ideally suited for the growth of large protein crystals since it reduces gravity induced convection (28, 3).

Considering that there are thousands, if not millions, of proteins of great medical and commercial value, if their structures are known, a major project has been launched, under the aegis of commercialization of space, to grow large protein crystals on space platforms. Also optical techniques have been successfully used in the space environment (4, 5, 6, 7).

One of the crucial steps in protein crystal growth is to detect the onset of nucleation and then certain steps have to be taken to change the physico-chemical nature of the solution in real-time. A generic technique sought for protein crystal growth would thus have the following components:

- Nucleation onset detector
- Detector signal processor
- Signal feedback to physico-chemical controllers

We have investigated various optical and signal processing techniques in the context of protein crystal growth as described above.

2. OPTICAL TECHNIQUES

Protein solutions are, in general, clear and transparent with an index of refraction close to that of water. Onset of nucleation, in principle, should manifest itself as local fluctuations in the refractive index. There are several optical techniques available to detect these fluctuations in the local refractive index.

These include:

- Direct microscopy
- Scattering
- Interferometric microscopy
- Ultra violet absorption
- Polarization
- pH Value

The latter being (so far) non-optical is still included in the list since the pH value seems to be a critical parameter in protein crystal growth. In a future study, the possibility of using pH value will be investigated. Direct microscopy has only limited value except for seeing the crystals after they have reached a certain size, typically 10 microns.
Direct microscopy in combination with polarization, say in the form of Nomarski microscope, may be a valuable tool since almost all bio-chemicals exhibit some optical activity. This subject is currently under study by us and will be reported later.

Light scattering is a very powerful technique to detect local fluctuations in the refractive index. Depending on the S/N ratio, however, samples over a certain region of the solution and gives a statistical average of the state of nucleation. A detailed study of light scattering and the extent of nucleation has yet to be carried out.

Ultra violet absorption, particularly in the region of 280 nm, is, again, a very powerful technique as it relates to protein concentration (8). It has been one of the traditional techniques used in protein crystal growth. A quantitative study, suitable for real-time closed loop control of the protein crystal growth, however, seems to be lacking.

Interferometry, as direct interferometry or in various guises, e.g. microscopic, schlieren, holographic, etc., has been extensively investigated and used in crystal growth studies. As a first step, we have used a commercial Interferometric Microscope (Leitz Metallograph; courtesy of the Center for Microgravity and Materials Research, UAH) to both directly view and examine interference patterns during the crystallization of Lysozyme protein.

In Fig. 1 the optical schematics of the interferometric microscope is shown.

Fig. 1. Interference Microscope

It is a traditional configuration suitable for white light fringes. The protein solution is held in a special holder as shown in Fig. 2.

Fig. 2. Protein solution holder.

A thin microscope slide is coated with a thin Au film on the 'bottom' side. On the 'top' side of the slide, a thin (0.8 mm) teflon washer is glued to form a thin cylindrical region to serve as solution containment volume. This cylindrical cavity is filled "to the brim" with the protein solution (previously mixed with the proper NaCl buffer solution). A thin microscope slide covers the entire cavity to furnish a flat parallel region of the solution suitable for interferometric observation. The entire 'slide assembly' is mounted on a 'Hotstage' for eventual temperature control (Courtesy Dr. E. Meehan, Chemistry department, The University of Alabama in Huntsville.) as shown in Fig. 3.
The sample holder slide rests at the center and the outer edges on annular metallic supports which can be heated or cooled to generate radial temperature gradients. By choosing the proper temperature profile it should be possible to position the region of crystallization under the microscope for optimum viewing.

A simple slide mechanism provides the facility for viewing either the interferometric pattern or the direct image of the sample. Furthermore, the orientation of the fringes can be changed by using the micro-manipulators of the microscope stage. This proved very helpful in a related attempt to identify and determine the face angles of protein crystals.

In Figs. 4 and 5 the growth stages of a crystal are shown.

The nucleation site can be easily identified as abrupt changes in the fringe curvature. Fig. 6 shows the distinct fringe pattern from the protein crystal faces.
Fig. 6. Fringe pattern from Protein crystal faces.

By measuring the relative orientation of these fringes it is possible to determine the relative orientation of the crystal faces in situ.

3. ACKNOWLEDGEMENTS

The authors are grateful to the Center for Microgravity and Materials Research for providing their facilities. R. F. Xiao has been most helpful in realizing the optical set up. Dr. E. Neehan and Pam Twig of Chemistry Department offered their generous help in furnishing Protein Solutions and their general expertise.

4. REFERENCES


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* Work supported in part by NASA Research Grant No. NAG8-599.

** Visiting Graduate Assistant from Ecole Normale Superieur de Telecommunication, Paris.