

## Detection of Early-Stage Protein Crystallisation using DPI

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### Introduction

The three-dimensional structure of a protein, which is key to understanding its biological function and plays a critical role in drug design and discovery, is predominantly determined by X-ray crystallography. However, the growth of suitable protein crystals remains a major bottleneck in structural determination, as proteins are extremely hard to crystallise. Each protein has its own specific set of crystallisation conditions and no generic set of crystallisation rules exists. As a result, hundreds or even thousands of crystallisation trials must be performed on any target protein, of which less than 1% typically yields promising results.

This application note describes the use of Dual Polarisation Interferometry (DPI) to monitor the crystallisation of proteins in real time.

The interference fringe pattern generated by the dual slab waveguide **AnaChip™** (Figure 1a) used in Farfield **AnaLight®** instruments is characterised by a number of parameters, including its period and contrast (as defined in Figure 1b). The contrast is a measure of the amount of light guided in both waveguides, and as such is affected by any losses such as scattering that occur on the top (active) waveguide surface. It is the contrast parameter that is used to monitor protein crystallisation using DPI.

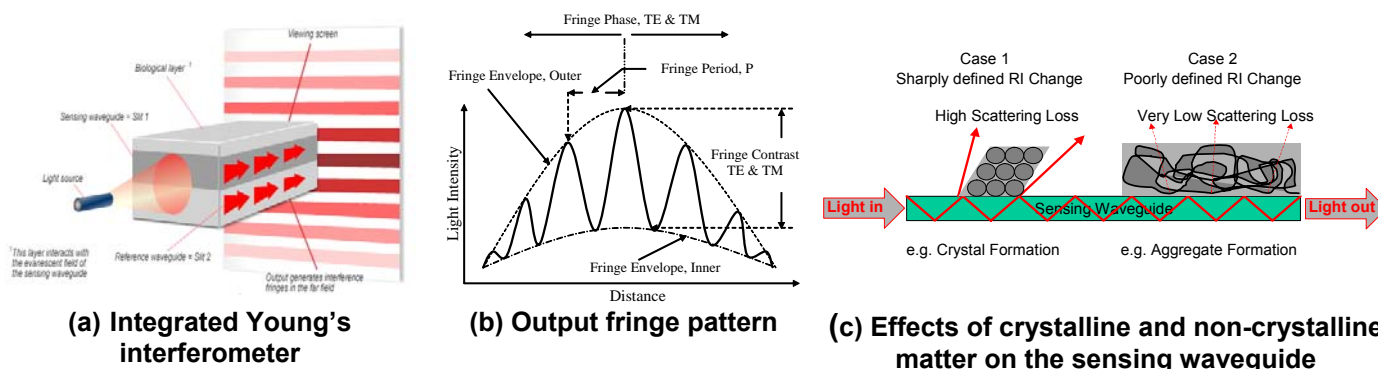


Figure 1: Crystallisation monitoring using DPI

### Experimental

The DPI experiments were performed on a Farfield **AnaLight®** instrument. The surface used was an unmodified silicon oxynitride **AnaChip™**. The temperature of the samples was controlled to 20°C throughout. Reagents were analytical grade or higher and water was high purity.

The precipitant solution was prepared a few minutes before the sample injection was made. Once the **AnaChip™** was fully covered with precipitant solution, the flow was stopped. In addition, the surface of the **AnaChip™** was monitored with a microscope operating in polarising mode mounted on top of the flow cell (above the **AnaChip™**). Only light caused by birefringence reached the CCD camera attached to the microscope.

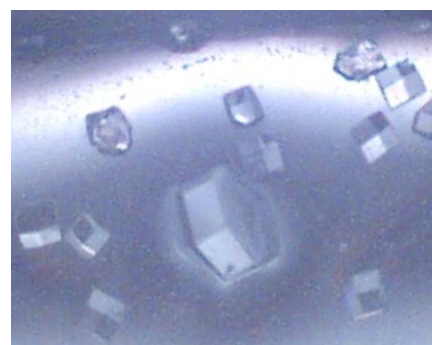
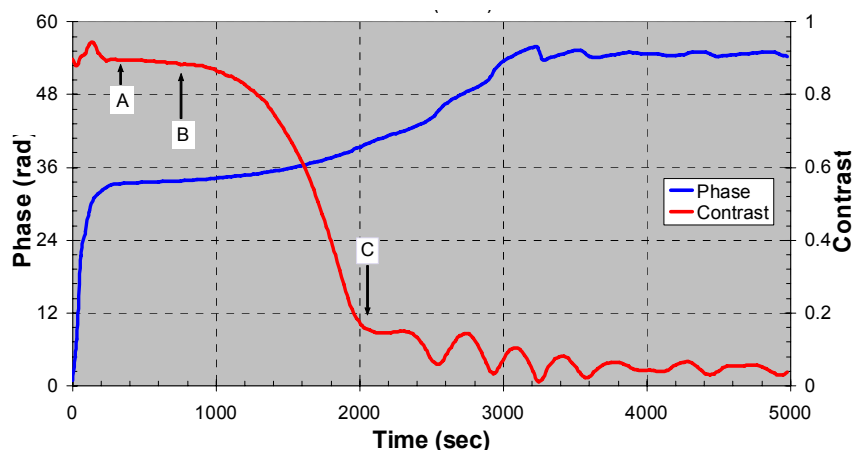
**Lysozyme Crystallisation:** The crystallisation of lysozyme was achieved by dissolving 80mg of the protein in 1.0ml of sodium acetate (aqueous, 50mM, pH 5.5) followed by addition of 1.0ml of NaCl (aqueous, 10% w/v) as a precipitant. Reducing the concentration of NaCl to 2.5% w/v inhibited the crystallisation of lysozyme and resulted in a clear solution.

**β-Lactoglobulin Crystallisation:** The crystallisation of β-lactoglobulin was achieved by dissolving 20mg of the protein in 1.0ml of sodium acetate (aqueous, 50mM, pH 2.5). No other reagents were added. β-Lactoglobulin precipitated under these concentrations.

**Results and Discussion**

**Lysozyme Crystallisation:** Figure 2 shows the effects of the formation of protein crystals on the DPI phase and contrast parameters. The phase increases as the protein adsorbs to the **AnaChip™** surface. However, as the protein forms a crystalline structure on the surface, light from the sensing waveguide is lost through scattering (as depicted schematically in Figure 1c) leading to the drop in contrast. A photograph of the lysozyme crystals grown on a **AnaChip™** is shown in Figure 3.

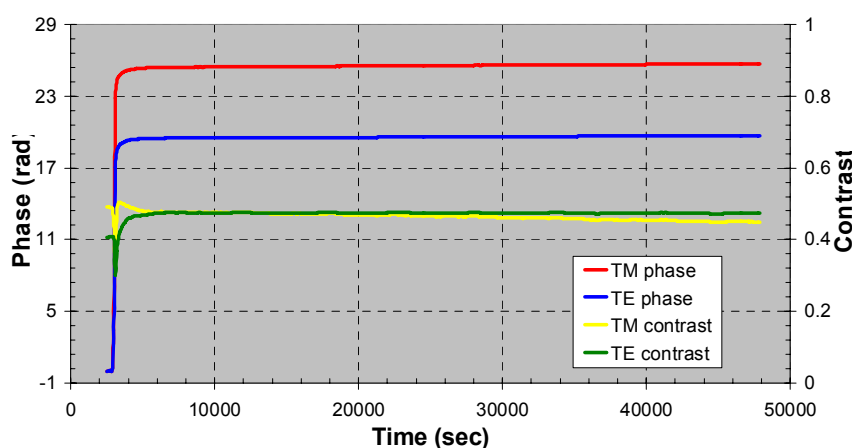
The decrease in the contrast parameter started approximately 8 min (point B) after the instrument flow was stopped (point A), and was complete in less than 30 min (point C). For eight experimental trials using lysozyme under crystallising conditions, DPI consistently predicted crystallisation more than an order of magnitude earlier than the birefringence technique (by computing the total intensity of the images captured by the CCD camera) could detect significant crystals.



**Figure 2: Effects of the growth of lysozyme crystals on the DPI phase and contrast parameters – see Table 1 for explanation of data labels A, B & C**

**Figure 3: Photograph demonstrating lysozyme crystals grown on an AnaChip™**

The contrast parameter remained unchanged in the cases where no crystallisation and no precipitation occurred for lysozyme (Figure 4). The phase increased initially due to the formation of an adsorbed protein layer, and subsequently remained constant throughout the experiment.



**Figure 4: DPI phase and contrast parameters for lysozyme under non-crystallisation and non-precipitation conditions**

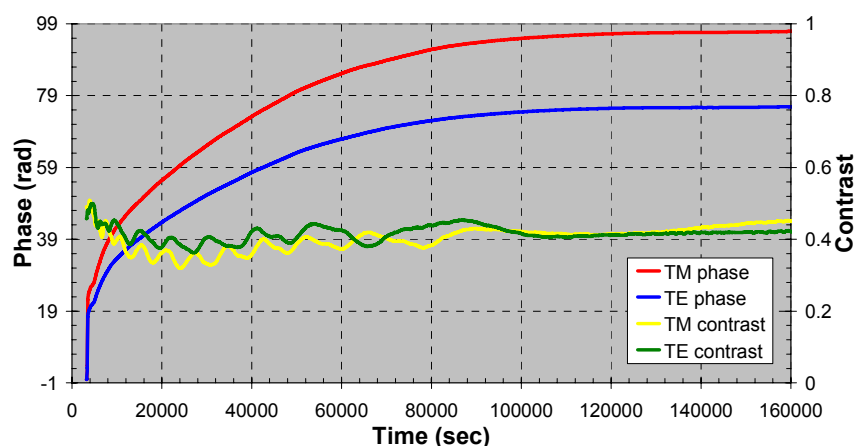
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The DPI technique enables the calculation of the refractive index, thickness and coverage of the protein layer adsorbed onto the surface of the **AnaChip™** during the first 30 min of the lysozyme crystallisation experiment in **Figure 2**. The results (see **Table 1**) indicate that crystallisation on the **AnaChip™** proceeds *via* an adsorbed protein layer averaging 6.7nm thick and increases to 10nm immediately before crystallisation. The results also show that the contrast loss occurred during the early stages of crystallisation, and could therefore be used to monitor the process and provide an early indication of the onset of crystallisation in protein crystallisation trials.

Measurement	After Flow stopped (A)	Onset of Contrast loss (B)	Before Total Collapse (C)
Layer RI	1.452 ± 0.008	1.455 ± 0.001	1.458 ± 0.007
Thickness (nm)	6.7 ± 0.7	8.9 ± 2.2	9.9 ± 2.1
% Coverage Erect	159 ± 28	214 ± 51	246 ± 54
% Coverage Prone	238 ± 41	320 ± 76	369 ± 81

**Table 1: Refractive index, thickness and coverage of the adsorbed lysozyme layer during the different stages of the crystallisation experiment (see Figure 2)**

**β-Lactoglobulin Crystallisation:** The response in the phase and contrast parameters to the precipitation of β-lactoglobulin on the surface of the **AnaChip™** is illustrated in **Figure 5**. The phase increased in response to the adsorption of a thin layer of protein as the solution was flowed over the surface, and then reached a maximum. The contrast displayed some low-magnitude and low frequency oscillations but, in general, also remained unchanged.



**Figure 5: DPI phase and contrast parameters for the precipitation of β-lactoglobulin**

The crystallisation of a range of common proteins such as myoglobin and glucose isomerase has also been studied using DPI, and similar results to those shown above were obtained. In addition, a MAP kinase crystallisation in the presence and absence of a known specific binder has been examined. In this case, crystallisation proceeds from a liquor containing precipitate. All results to date indicate that DPI can be used to differentiate between crystallisation events and non-crystallisation in proteins.

## Conclusions and Benefits

The **AnaLight®** instruments and their experimental protocols give the researcher a unique combination of high-resolution data in real time on the phase and contrast of the interference fringes generated by the DPI technique. DPI is an important enabling technique for protein crystallography researchers, giving them the ability to:

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- Distinguish easily between protein crystallisation and other macromolecular physical processes, such as precipitation and aggregation
- Detect crystallisation at the early nucleation stage, the onset of the crystallisation process
- Detect the onset of crystallisation 10-100 times faster than with polarised microscopy.
- Enable crystallisation processes to be monitored, and hence developed and optimised, in real time

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Telephone the applications team on +44 (0) 870 950 9717

*Note: The use of Dual Polarisation Interferometry for the detection of early-stage protein crystallisation is the subject of Patent Application PCT/GB02/02185*