Microfabrication of Sample Holders for Cryogenic Small Angle X-Ray Scattering and Flow Cells for Fluorescence Measurements of Ligand Diffusion in Protein Crystals

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Primary CNF Tools Used: Heidelberg DWL 2000 Laser Writer, Hamatech Wafer Processor, Filmetrics Reflectometer, SÜSS MA6 Contact Aligner, Oxford PlasmaLab 80+, SUEX Laminator, Harrick Plasma Generator, PDMS Casting Station, DISCO Dicing Saw, KOH Hood and Bath

Abstract:

Small angle X-ray scattering (SAXS) and X-ray crystallography are key tools used to study the structure and function of biomolecules. We have been developing microfabricated sample cell arrays for collection of SAXS data from biomolecular samples cooled to cryogenic temperatures, which could simplify SAXS sample handling and increase data collection throughput. We have also been developing microfluidic flow cells for quantitative fluorescence measurements of diffusion of small molecules into biomolecular crystals. These measurements inform choice of crystal sizes in time-resolved crystallography of biomolecules in action.

Summary of Research:

Sample cell arrays for cryo-SAXS. Small-angle X-ray scattering (SAXS) probes the shape and size of biomolecules (e.g., proteins) in solution. SAXS is currently performed using biomolecule samples at or near room temperature. Samples are rapidly damaged by X-rays and may undergo degradation during transport to the X-ray source. If samples are cooled to cryogenic temperature, the rate of X-ray damage decreases by orders of magnitude and samples can be stored indefinitely without degradation. Our goal is to develop sample cell arrays and methods to enable routine high-throughput SAXS sample handling and measurements at cryogenic temperature. Major challenges in achieving this goal include finding a combination of materials, structures, and protocols that allow easy loading of nanoliter sample volumes, sample cooling into a vitrified state without formation of crystalline ice, bubbles, anisotropic stresses, or fractures, and that allow quantitatively and qualitatively identical data to be collected from any cell in the array.

Our sample cell arrays are constructed from two 3 mm × 12 mm pieces of 300 μm thick, <100> oriented, double-side polished Si wafer, coated via LPCVD with 500 nm of SiN. The wafer is photolithographically patterned, SiN is removed from one side, and Si is removed using an anisotropic KOH etch, leaving arrays of 500 μm square, X-ray transparent SiN windows. A sheet of SUEX is bonded to the window side of the wafer and patterned to define cell alignment and liquid trapping features. The wafer is diced into rectangles containing eight windows, each forming half of a sample cell array. On one half, segments of thin-walled polyimide tubing are bonded over each nitride window and quartz spacers are attached. The spacers fix the distance between nitride windows in the two halves of the device, fixing the X-ray path length through each cell.

We have evaluated our cryoSAXS sample cell arrays at synchrotron beamlines at CHESS (Cornell) and NSLS-II (Brookhaven National Lab). The cells allow rapid cooling to a vitrified sample state. The critical challenge has been to eliminate all sources of cell-to-cell variability/irreproducibility in SAXS scatter, as differences as small as 1% can have a major impact on data quality. We have been addressing this challenge via changes in device geometry and assembly, changes in X-ray beamline configuration, and changes in device cleaning protocols.

Our current design — involving both microfabrication and precision manual assembly — presents obstacles to both reproducible data collection and routine, high-volume production and use. We will continue optimization of the current design and exploring alternative designs, including all-microfabrication designs made possible using two-photon polymerization.

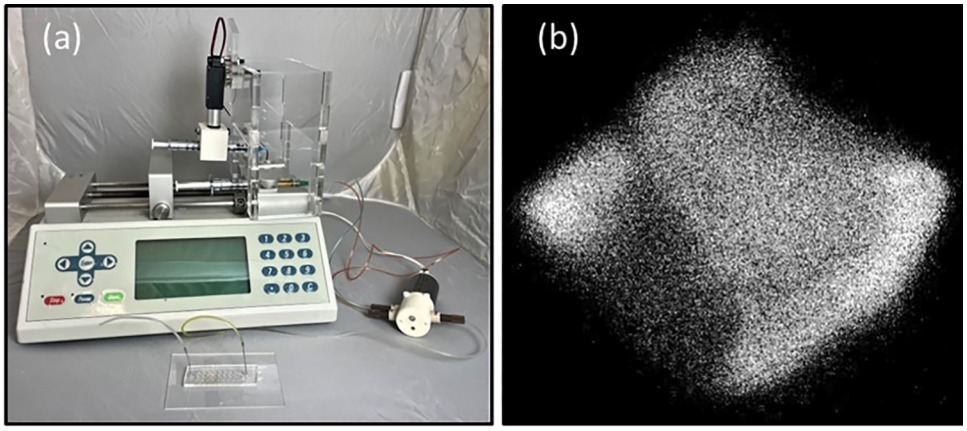

Figure 1: (a) Experimental setup including microfluidic crystal trap array for measuring diffusion of fluorescent small molecules into biomolecular crystals. (b) Two-photon fluorescence image of a fluorophore (lysotracker blue DND-22) diffused into a ~ 50 µm lysozyme crystal.

Microfluidic traps for *in crystallo* diffusion measurements. A long-term project in structural biology has been to develop methods for making atomic-resolution "movies" of biomolecules in action. This typically involves mixing small crystals with a ligand that diffuses into the crystal and binds to the biomolecule, reacting with it and/or triggering a change in its structure. X-ray diffraction patterns are recorded at different times after the start of mixing and are used to generate "snapshots" versus time.

We demonstrated a mix-and-quench method [1] where biomolecular crystals on a support are translated through a film containing reactant. After a delay, the crystals are plunged into liquid nitrogen, stopping the reaction and trapping the structural state for subsequent X-ray examination. We achieved a nominal time resolution of 40 ms, and with hardware improvements sub-5 ms time resolution should be feasible.

However, a key unknown is the time required for a given ligand/reactant to diffuse into a given biomolecular crystal. Time-resolved experiments have so far assumed that diffusion constants within crystals are the same as in free solution. However, biomolecular crystals are nanoporous solids with complex internal pore geometries. Diffusion constants could be one to two orders-of-magnitude smaller, causing uncertainty in reaction "time points" due to inhomogeneous ligand distribution within the crystal, limiting achievable time resolution, and/or requiring use of much smaller crystals.

To examine diffusion within crystals, we are fabricating microfluidic flow cells for use in multiphoton imaging (Figure 1). Crystals in carrier solution flow into the cells and are trapped at an array of locations, and then a solution containing a small-molecule fluorophore flows into the cell and diffuses into the crystals. To create these crystal traps, we fabricate a master mold by bonding a SUEX sheet to a silicon wafer and photolithographically patterning it. Trap arrays are fabricated by casting a 2 mm layer of PDMS on top of the wafer. Once the crystal traps are filled by a flow of crystal-containing solution, fluorophore is abruptly introduced using an electronically controlled syringe-holding manifold and a switching valve.

We have demonstrated successful trapping of 50 μm crystals and obtained fluorescence data yielding a plausible initial diffusion coefficient estimate. We are improving the design to allow study of larger crystals (which should give more accurate estimates) and faster flow switching to improve time resolution.

References:

[1] Clinger, J. A., Moreau, D. W., McLeod, M. J., Holyoak, T., and Thorne, R.E. Millisecond mix-and-quench crystallography (MMQX) enables time-resolved studies of PEPCK with remote data collection. IUCrJ 8, 784-792 (2021).