# MINIATURIZED AND HIGHLY PARALLEL PROTEIN CRYSTALLIZATION ON A MICROFLUIDIC DISC

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## ABSTRACT

For the first time we present a new microfluidic system for miniaturized and highly parallel protein crystallization experiments by the free interface diffusion (FID) method. The novel system is based on a microfluidic disc fabricated by hot embossing which features 100 protein crystallization chambers enabling up to 100 different crystallization experiments in parallel. The microstructures exhibit minimal feature sizes of 30 µm and a maximum aspect ratio of 1. The fluidic design of the disc enables lamination of nanoliter volumes of protein and crystallization solution (precipitant) in a crystallization chamber of minimum volume of 5 nL. The protein sample is loaded to the disc by a non-contact nL-dispenser with a minimal dosage volume of 1 nL and dead volume of only 500 nL. All liquid processing steps on the disc are accomplished by centrifugal forces caused by rotation of the disc. Up to 80 µm large crystals of catalase, lysozyme, proteinase K and insulin, have been produced on the disc to demonstrate the proper performance. Subsequently the crystals have been analyzed *in situ* in an X-ray experiment without removing them from the disc.

## INTRODUCTION

Structural Biology is one of the fastest growing fields in current life science research. In order to determine the three-dimensional structure of biological macromolecules and their complexes X-ray crystallography is by far the most common technique, contributing to about 85% of the structural data in the Protein Data Bank (http://www.rcsb.org/pdb) [1]. A bottleneck specific to macromolecular crystallography is the necessity to grow well diffracting crystals from solubilized material [2,3], a process that is generally unpredictable for a particular macromolecule.

For this reason, different molecular constructs of a target molecule have to be tested against a large number of crystallization solutions (several hundred at different temperatures) in order to identify conditions that yield well ordered crystals. As a consequence, several high-throughput crystallization facilities [4,5,6] have been established over the past years which have automated the manual set up of experiments and reduced the demands on sample volumes. Because of significant financial and spatial demands of these facilities, there is a strong demand for alternative approaches. In this context microfluidic technologies can offer the advantages of very small sample consumption (~ 1 nl), reduced time to results and a compact design (lab-on-a-chip) at affordable costs.

Especially in microdimensions the FID method for protein crystallization has some intrinsic advantages over the vapor diffusion method predominantely applied in the high throughput crystallization facilities. Though FID has been recently demonstrated in nanoliter volumes [7] most methods proposed for miniaturized crystallization exhibit a lack of flexibility and suffer from large dead volumes. The approach presented in the following is based on the flexible use of hot embossed, low-cost microfluidic substrates and a low-volume dispensing system with a very low dead volume of only 500 nl for the partly very precious protein solutions. The microfluidic substrate can be easily mounted in an X-ray beam for *in situ* examination of the intrinsic diffraction quality of crystals, avoiding potentially damage by direct handling of the crystals.

## PRINCIPLE AND DESIGN

Core of the presented system is a transparent, polymeric microfluidic substrate (disc) on which protein crystallization can simultaneously take place in up to 100 crystallization chambers. The crystallization assay is assembled by centrifugal forces. Therefore a specially designed „disc player“ was developed to process the disc. The protein solution is loaded onto the disc by a PipeJet nanoliter dispenser [8] , which is integrated in the disc player. Figure 1 shows a schematic of the system setup.

In a FID crystallization experiment protein sample and precipitant at different volume ratios have to be brought into contact to form a defined liquid/liquid interface from which a concentration gradient is estab-

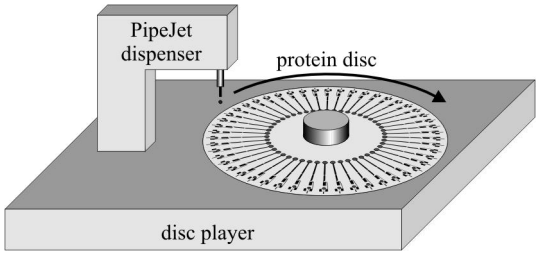


*Figure 1: Schematic of the system setup*

lished over time during the incubation. The lamination of the liquids is accomplished on the discs in so called crystallization chambers whereby each of the chambers has one inlet and one outlet (see fig. 2). After all samples have been loaded onto the disc, first the protein samples are centrifuged into the chambers. Hereby the volume of the protein sample is defined by the PipeJet dispenser delivering the protein solution as free flying droplet to the disc. The dispensed protein sample volume is adjustable from 1 nl to 20 nl. The volume of the precipitant entering the chamber directly after the protein is defined by the remaining free space in the chamber. Excess precipitant solution is discharged into a waste chamber. Thus, the ratio between protein sample and precipitant is controlled by the volume of the protein sample and the total chamber dimensions. Figure 2 illustrates this principle.

In contrast to the predifined volume of proteins the precipitants are pipetted manually or by standard pipetting robots in volumes of 0.5 µl into the corresponding reservoirs. Because only a small amount of this volume is needed, microfluidic structures were implemented on the disc which meter the required precipitant volume or transport excess liquid into waste chambers.

Since the precipitant's volume and mass are up to 500 times higher than for the protein, the precipitant is much stronger affected by the centrifugal forces. Therefore different microfluidic structures had to be implemented which decrease the flow of the precipitant and take care that it enters the chamber after the protein. Two microfluidic structures, type A and type B were evaluated (see fig. 3). In the type A structure, the microfluidic element MFE_1 represents a reduction of the microchannel cross-section to increase the flow resistance which results in a reduction of the volume flow for the precipitant solution. MFE_2 consists of a chamber with an inlet and an outlet on its top. The chamber has to be filled completely before the liquid can leave via the outlet. This leads to a delay arrival of the precipitant in the crystallization chamber compared to the protein. After complete filling of the crystallization chamber, the excess precipitant solution is collected in waste chambers. The small width (30 µm) and height (30 µm) of chamber inlet and outlet act as diffusion barriers and seal the chamber from the waste.

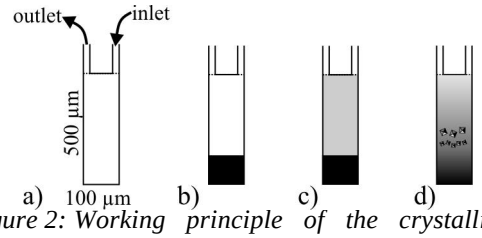


*Figure 2: Working principle of the crystallization chamber a) geometry b) protein sample is centrifuged into the chamber c) precipitant is centrifuged on top d) mixing by diffusion and forming of protein crystals.*

In the type B structures a well defined amount of precipitant is metered by the microfluidic element MFE_3 (see fig. 3b) [9] before lamination. This way both solutions are centrifuged in pre-defined volumes into the chamber. Immiscible, also pre-loaded silicone or paraffin oil is applied in this case to seal the protein-precipitant sandwich and to prevent evaporation.

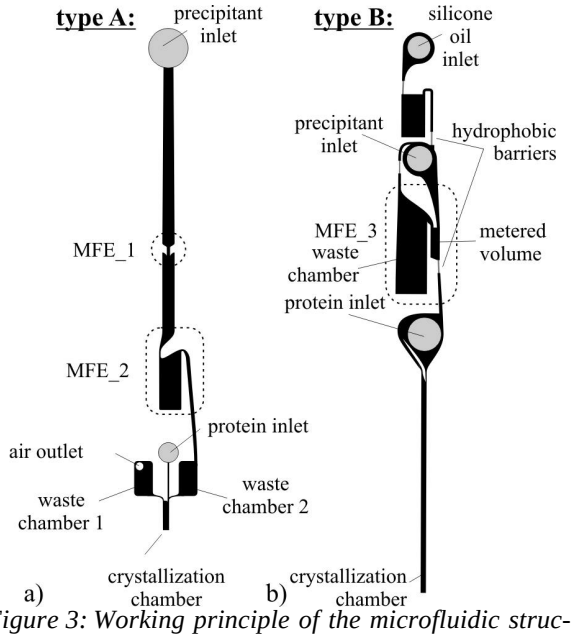


*Figure 3: Working principle of the microfluidic structures a) type A:MFE_1 & MFE_2 decrease the volume flow of the precipitant b) type B:MFE_3 meters a well-defined volume from the preloaded precipitant [9]*

## FABRICATION

**Microfluidic disc:** Basically three casting steps were required to obtain the final disc made from the polymer COC (see fig. 4). First an original mold was fabricated in silicon by Deep Reactive Ion Etching (DRIE) (fig. 4a) [9, 10]. Afterwards the silicon master was casted by a soft elastomer (Wacker Elastosil RT 607) (fig. 4b). Due to its elasticity the elastomer mold could not be used as embossing tool as described in [9, 10] in the present case. It deformed during the final hot embossing process. Hence one more copy was casted from it using a more robust temperature stable epoxy material (Weicon Type C Plastic Metal) (fig. 4c). Finally this epoxy mold was hot embossed into the polymer material COC (fig. 4d).

In the current production process the embossed COC disc had to be post processed by milling and drilling to obtain a round disc with a centered hole and the sample inlets. In a mass production obviously all features would be realized within one embossing or injection molding process. The open structures of the disc were covered by a self adhesive film (Polyolefin sealing foil, HJ Bioanalytik) typically used for sealing micro titer plates.

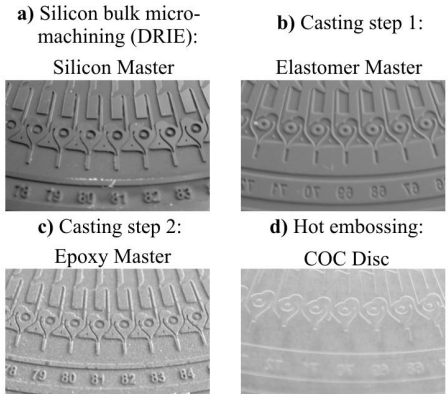


*Figure 4: Different stages during disc fabrication a) original mold fabricated in silicon by DRIE b): elastomer mold c) epoxy mold d) final disc in COC*

**Disc player:** A picture of the specially designed disc player is shown in figure 5. It basically consists of a stepper motor (Nanotec, ST4018M1804) to rotate the disc and a PipeJet dispenser for protein dosage into the disc (BioFluidix, PipeJet R2b). Below the PipeJet dispenser a VGA CCD camera was integrated for optical control of the protein sample dispensing event. On top of the motor rotor a CD holder is mounted. Finally a removable protective cover with integrated temperature and air humidity sensors can be placed on top of the player to provide controlled environmental conditions. The player is connected to a computer via USB to control dispensing and disc rotation by a dedicated Visual Basic.NET program with graphical user interface.

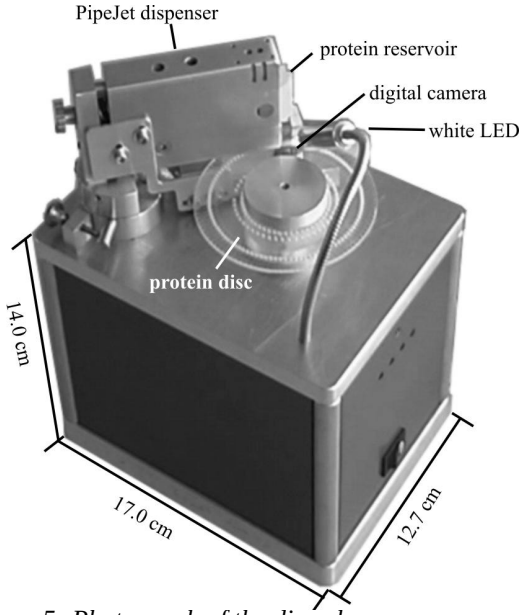


*Figure 5: Photograph of the disc player.*

## EXPERIMENTS AND RESULTS

The experimental work was divided into three sections. First experiments showed that protein and precipitant can be laminated accurately onto each other with an initialy sharp interface that diffuses over time. Second, different protein samples were crystallized using the approach and hardware as described before. Finally the crystals were analyzed inside the CD in an x-ray beamline.

**Lamination of protein and precipitant:** Ink and water were used as samples to demonstrate that both solutions are laminated onto each other properly. Black ink represents the protein sample and water the precipitant (see fig. 6). During the these preliminary experiments

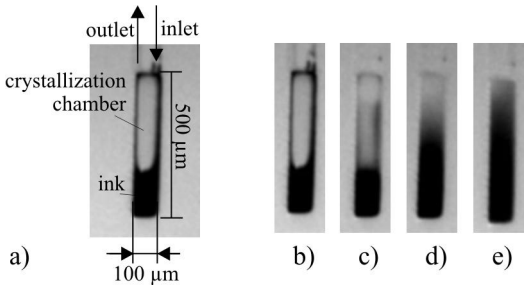


*Figure 6: Water and black ink laminated onto each other in the crystallization chamber a) geometries b) ink only c) water laminated onto ink d) & e) the initially sharp interface diffuses over time (c to d: 15 min)*

no pre-loading of the precipitant was applied. First 2 nL black ink was dispensed into the protein inlet and subsequently centrifuged at 5000 rpm into the crystallization chamber (fig. 6b). Then the player was stopped, water was filled into the precipitant inlet and centrifuged into the chamber (fig. 6c). Afterwards the diffusion process was observed with a microscope (fig. 6d&e). The results show that both liquids are laminated in an accurate way onto each other and diffuse subsequently as desired. The edges wetted with black ink, visible in fig. 6a originate from stopping the player before water was pipetted into the precipitant inlets. If the player is stopped, capillary forces draw the black ink along the edges of the crystallization chamber towards inlet and outlet and degrade the sharp interface. Therefore the precipitant has to be pre-loaded onto the disc to avoid stopping of the player and to keep the interface of the protein solution always flat.

First experiments with pre-loaded samples showed that the integrated microfluidic elements insufficiently delayed the flow of the precipitant so that it entered the crystallization chamber before the protein. For the type A structures this resulted in a complete filling of the crystallization chamber with precipitant. The protein was finally mixed with it in the waste chambers where the protein crystallization took place in an uncontrolled way (fig. 7). In the type B structures the precipitant was metered, therefore the crystallization chamber did not overflow and the protein was subsequently laminated properly onto the precipitant. Simply the order of protein and precipitant were interchanged in the crystallization chamber compared to the intended process.

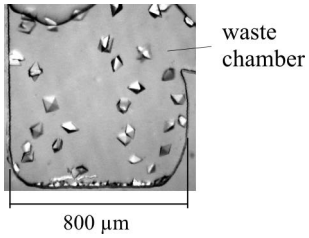


*Figure 7: Proteinase K crystals in the waste chambers*

Since this does not affect the crystallization process the structures of type B were used for further experiments.

**Protein crystallization:**

All protein crystallization and beamline experiments were performed at the high-throughput crystallization facility at the EMBL, Hamburg [4]. Crystallization of lysozyme, catalase, proteinase K and insulin were chosen as test cases. Crystallization of all of these proteins was successfully demonstrated applying the described method and hardware (see Figure 8).

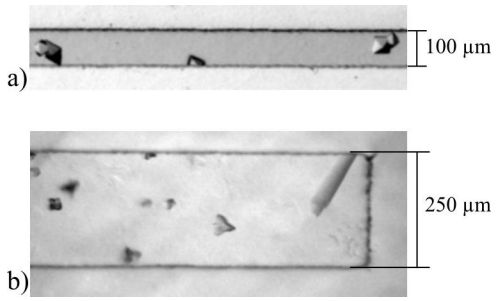


*Figure 8: Crystals of proteinase K (a) and catalase (b) by free interface diffusion in crystalization chambers.*

**Protein structure analysis in the x-ray beamline:**

As shown in figure 9a the microfluidic substrate was placed *in situ* into the x-ray beamline. The diffraction results were analyzed with the commonly used DENZO software. Figure 9b shows the software results if the CD is placed in the beamline with no crystals present. No diffraction peaks were detected. After moving the disc until a proteinase K crystal was positioned in the beam, pronounced peaks could be observed (fig. 9c).

## CONCLUSION

The presented technology enables miniaturized protein crystallization experiments in a highly parallel manner with higher flexibility than current systems. The extremely low crystallization volume and dead volume lead to better use of protein samples and reduced costs. With 600 nL of total protein solution 100 experiments using 100 arbitrary precipitants can be performed simultaneously on the presented disc. Furthermore, due to their design, size and material selection the discs can be easily transported and analyzed *in situ* after incubation.

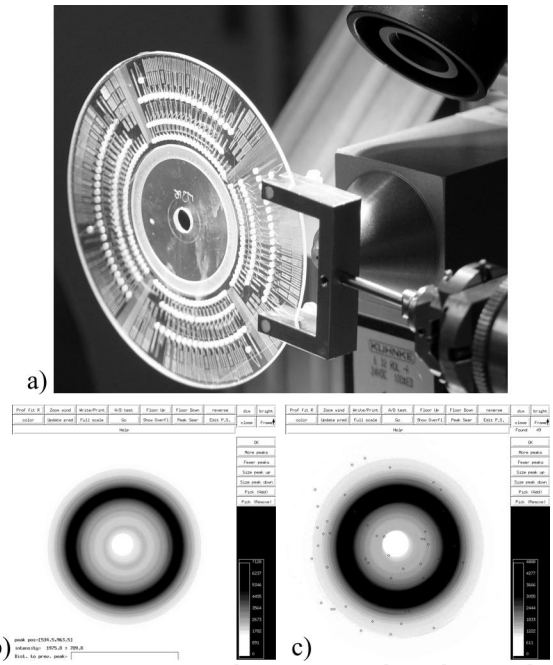


*Figure 9: Structure analysis in x-ray beamline a) disc placed in beamline b) results without crystals present c) results for proteinase K crystal*

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