# OPEN SURFACE BATCH CRYSTALLIZATION OF PROTEINS ON AN AUTOMATED NON-CONTACT nL-DISPENSER SETUP

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## ABSTRACT

We present a novel protein microbatch crystallization approach for screening applications in the low nL-range with no necessity for any microfabrication in advance or subsequent cleaning step. Crystallization solution with sample consumption of 5 nL per trial is applied by non-contact direct injection into paraffin oil predeposited as 150 nL droplets upon planar, non-structured hydrophobic substrates of selectable dimension. Consequently, an almost arbitrary number of crystallization trials with trial densities of up to 50 per cm² can be conducted. Successful crystallization of lysozyme on hand-cut polymer snippets is demonstrated both by premixed crystallization solution and mixing in oil.

**KEYWORDS:** microbatch protein crystallization, planar substrate, non-contact droplet injection

## INTRODUCTION

In-depth understanding of proteins demands detailed knowledge of their 3D structure mainly enabled by x-ray crystallography. However screening for suitable crystallization conditions requires hundreds to thousands of single trials consuming up to multiple mL of laboriously obtained protein solution. Within the last decades a rising number of microfluidic tools have reduced the required sample volumes to a few hundred nL per run using nano-well [1] or microcapillary-based [2] chips of glass or PDMS. While microfabrication of such chips was facilitated most protein scientist won't have the opportunity to design or build up their own chips or even use them as long as they are not commercially available.

With the presented approach, nL-droplet protein crystallization is carried out upon virtually every planar (disposable) substrate with commercial dispenser heads obviating any need for subsequent cleaning.

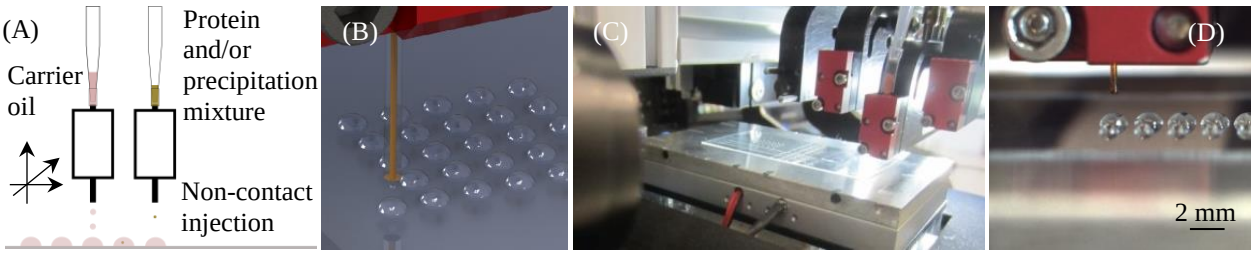


*Figure 1. (A) Schematic drawing of open surface batch crystallization process and robotic setup. PipeJet modules with pipe-attached disposable reservoirs are primed with carrier oil & crystallization solution. Carrier oil is deposited first followed by sample-injection both in a non-contact manner. (B) Rendered close-up of pipe-based sample injection procedure. (C) Temperature-controlled substrate surface with axis mounted multi-dispenser rack placed above. (D) Close-up of single dispenser module with 200 µm (i.d.) pipe during injection.*

## EXPERIMENTAL

Droplet generation was carried out with piezo-actuated nL dispenser modules (PipeJet®, Biofludix, Germany) [3] using disposable pipes (Fig. 1). Multiple dispenser modules covering the handling of oil, protein solution and crystallization buffer are mounted on an x-y-z stage (+/- 30 µm) equipped with two high-resolution cameras for process observation and dispenser alignment. A customized substrate holder

(Fig. 1, C) features peltier-based surface cooling, substrate vacuum aspiration and dispenser-to-surface leveling. Substrates are dip-coated with Teflon AF 1600 (Dupont, USA) to increase static contact angle of carrier oil. Crystallization trials are generated by sequential dispensing of carrier oil droplets followed by direct injection of premixed crystallization solution or mixing of protein solution and buffer by two-step injection. Chemicals and samples were purchased from Sigma-Aldrich (Sigma-Aldirch, Germany). All hardware was integrated into a custom-built control software written in VB.NET.

Droplet injection parameters and impact of dispenser alignment were studied on a stroboscopic camera setup (Fig 2.). Evaporation analysis was done by long-term microscopic observation and microgravimetry on a customized setup using a Mettler Toledo XP2U (Mettler-Toledo, Germany) balance.


*Figure 2. Dispenser alignment validation from stroboscopic setup. Each subframe represents a series of single droplet injection: before-after-subtraction. A closed white semicircle indicates complete injection.*

**RESULTS AND DISCUSSION**

Medium viscous paraffin oil exhibited best sealing abilities yielding about two times lower evaporation rates of aqueous droplets compared to mineral oil. On using crystallization solution-to-oil ratio of 1:30, stable batch crystallization screening is possible within several days to one week without further reduction in evaporation rate. Still, a cover oil with even lower permeability would be advantageous as it would allow for long-term crystallization.

Paraffin carrier oil droplets at volumes of 150-300 nL per trial were dispensed on Teflon-coated substrates exhibiting a contact angle of 67 ± 3.4° (110 ± 2.0° for aqueous solutions) with densities up to 50 droplets/cm² and frequencies of 6-10 trials/min (including injection). No inconsistency in crystallization trial generation was observed using various Teflon-coated substrates like glass or plastics.

We found that successful injection of crystallization solution (15 mg/ml protein) down to 5 nL is achieved at droplet velocities of 0.5-2 m/s. Measuring droplet velocities at injection distances from 1.5 mm to 4.5 mm did not show a significant reduction in kinetic energy. Nevertheless, the impact of misaligned dispenser or inclined droplet trajectories rises with injection distance. A precise x-y alignment thus is the most crucial step in advance to increase successful droplet injection. Faulty alignment in turn results in droplet bouncing or partial injection (Fig. 2, offset in sight) similar to the findings made by Sun et al. [4]. As a consequence of non-centered injection, we observed droplets that seem to swim between the oil and gas phases followed by floating to the lower edge of the carrier oil droplets and evaporation within minutes. In addition, deviating from the model of Sun et al., in-oil fusion of sequentially injected aqueous droplets may lift the resulting droplet to the outer border of the carrier oil droplet also generating contact to surrounding air (Fig. 3, D). With the setup as presented, successfully injected centered droplets are obtained at rates of >70 %.

Using standard crystallization conditions for egg white lysozyme (30 mg/ml stock solution) and premixed crystallization solution, crystallization frequencies of 33 % at evaporation rates of 0.5-2 nL/d (room temperature) were achieved (Fig. 3). Assuming adequate penetration depth of aqueous droplets into oil, varying the ratio of oil-to-protein solution might be used to optimize crystal size or quality tuning growth rate. Moreover, we were able to successful merge droplets of protein solution and crystallization screening buffer within carrier oil droplets using lysozyme (Fig. 3, C) yielding similar crystallization parameters as mentioned above.

The relatively low crystallization frequency might have several reasons. On the one hand non-centered droplets evaporate with higher rates yielding precipitation instead of protein crystals. On the other hand

the reduction in volume decreases the likelihood of nucleation simply by less particles (impurities) per volume. In fact we mostly found single crystals in our 5 nL droplets nearly agreeing with Zhu et al. [1].

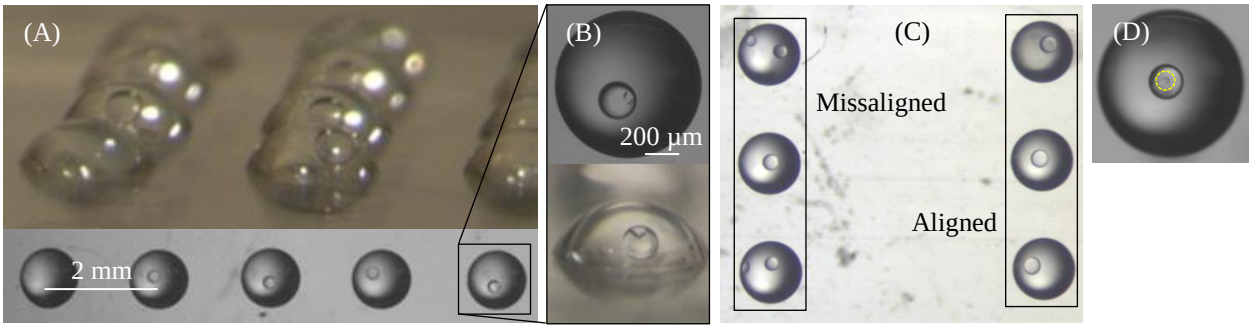


*Figure 3. Crystallization arrays of lysozyme in paraffin oil upon a hand-cut Teflon-coated COP substrate. (A) Side view & top view strip of open substrate crystallization array: 5 nL of 30 mg/ml lysozyme in 0.05 M NaAc, pH 4.6 injected into 150 nL paraffin oil each. (B) Successfully crystallized lysozyme. (C) Failed (left) and successful (right) sequential injection of protein solution & crystallization buffer. (D) Air contact after aqueous droplet fusion in oil (circle).*

## CONCLUSION

With the presented approach in hand we were able to demonstrate nL-droplet protein crystallization bypassing any microfabrication step in advance. Although current performance is lower compared to previously presented setups, the approach might overcome this drawback by its intrinsic flexibility using disposable pipe-reservoir combinations and most simple substrate processing.

In future work, the use of pipes smaller in diameter could further increase the number of trials per cm² while ongoing automation and software optimization will decrease processing times to improve throughput. However, as demonstrated earlier [1], further reduction in volume is not limited by technical constrains rather than the probability of nucleation events.

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