

Acoustic Liquid Handling Applied to Protein Crystallography—Miniaturizing, Formulating, Transferring, Seeding, Monitoring, & LCP Formation

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Abstract

Acoustic liquid handling—using sound to move fluids and suspensions—has been widely accepted in the pharmaceutical high-throughput screening community and now has been applied to protein crystallography processes. The technology uses low-energy pulses of sound to eject fluids from an open source eliminating any physical contact with the fluid—no pipette tips, no probes, no “whiskers.” Transfer volumes start at nanoliter levels but rapid repetition rates easily allow larger volumes to be transferred. We will show the latest results in five different areas relevant to crystallography:

1. Transfer of a wide range of fluids of varying viscosities and surface tensions, identical to or representative of solutions typically used in protein crystallization.
2. Formulate microvolumes of intermediate solutions through spot-on-spot placement.
3. Transfer of microcrystals for subsequent X-ray analysis or seeding experiments.
4. Non-invasive monitoring of protein crystallization fluids
5. Transfer lipidic cubic phase components to facilitate the self-assembly of nanoliter-scale environments for membrane protein crystallization.

We believe that acoustic liquid handling provides crystallographers with a new tool of multiple dimensions that can be used in many different crystallographic applications.

INTRODUCTION

The Labcyte Echo® 500 series revolutionizes liquid handling by using acoustic energy coupled with automatic calibration to eject fluids². The Echo 500 series allows for assay miniaturization to previously unattainable volumes. Echo liquid handlers transfer 2.5 nL droplets repeatedly, so precision and accuracy are consistent over a larger volume range³. Large volume transfer is achieved by transferring several hundred droplets per second. Transfer is non-contact and tipless, with increased cost savings from elimination of pipette tip costs and washing fluids.



The Echo Liquid Handler

Figure 1: The Echo liquid handler moves fluids without ANY physical contact with sample. A transducer moves beneath the plate holding the fluids and emits acoustic energy. The acoustic energy is focused at the meniscus. Droplet volume is controlled at 2.5 nL. Larger volumes are obtained by transferring more droplets. The transducer automatically changes position in order to keep the focus of the acoustic energy at the fluid surface.

EXPERIMENT 1:

Transfer of a wide range of fluids of varying viscosities and surface tensions, identical to or representative of solutions typically used in protein crystallization.

Experiment 1A: Transfer of Glycerol Solutions; Measurement of Accuracy and Precision

Transfer of glycerol solutions (0 - 60% by volume) was carried out on an Echo 555 liquid handler. The volumetric accuracy and precision was confirmed using glycerol solutions that were doped with fluorescein and the fluorescence emission was determined on a microplate reader.

Methods

Solutions of glycerol (0 - 60% by volume) in water were doped with sodium fluorescein to a final concentration of 0.15 mM and used to generate a reference dilution. Each concentration of glycerol was loaded into each well of a 384-well polypropylene Echo® qualified source plate (P/N P-05525; Labcyte Inc.) at volumes ranging from 20 - 50 μ L. Using the Echo 555 liquid handler, 50 nL volumes of varying glycerol solutions were transferred from the source plate to each well of a 384-well destination plate (Greiner bio-one). 50 μ L of 10 mM NaOH solution (pH 12) were added, the plates were centrifuged, incubated for 30 minutes at room temperature, and read on a 2100 EnVision plate reader (PerkinElmer). Relative fluorescence unit (RFU) values were converted to volumes against a standard curve of glycerol.

Results

The experimental results are depicted in a graphical format in Figure 2. Each bar represents data from 384 transfers (n=384) using the Echo 555 liquid handler. The columns in Figure 2 show that the average accuracy is 103% of the target transfer volume of 50 nL. The line in Figure 2 shows the average transfer precision. These experimental results indicate that acoustic transfer using the Echo liquid handler is robust for solutions of glycerol from 0 - 60% by volume, which is representative of transferring solutions of varied viscosity in the range of 1 - 15 cP.

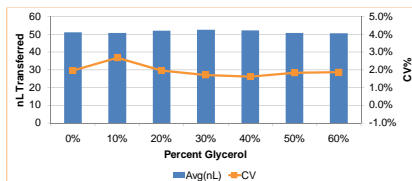


Figure 2: Transfer volume accuracy and precision of glycerol solutions doped with 0.15 mM sodium fluorescein as a function of % glycerol (v/v). The bars indicate the actual volume transferred when a nominal volume of 50 nL was requested. Maximum deviation from nominal was <5.5%. The orange line shows the coefficient of variation (CV). The CV ranged from 1.6% to 2.7%.

Experiment 1B: Transfer of 98 commonly used protein crystallography liquids

Transfer capability of the Echo 555 liquid handler was tested with two sparse matrix additive screens purchased from Hampton Research (Crystal Screen and Crystal Screen 2, Aliso Viejo, CA).

Methods

30 μ L of each fluid was placed in an Echo qualified 384-well polypropylene source plate. The plate was loaded into an Echo 555 liquid handler, and 50 nL of each fluid was transferred to a 384-well, clear-bottom polystyrene microplate. All transfers were performed using a single machine calibration with automated, dynamic power adjustment that covered all fluid types without the need for user intervention.

Results

Coalesced droplets from 9 destination wells were photographed and visually assessed for transfer performance (see Figure 3). All 98 protein crystallography standard solutions transferred to the destination well.

Over 1,400 protein crystallization fluids have been successfully transferred with an Echo 555 liquid handler. All of the transfers of crystallization fluids were made with the same calibration. Contact the authors for a complete list.

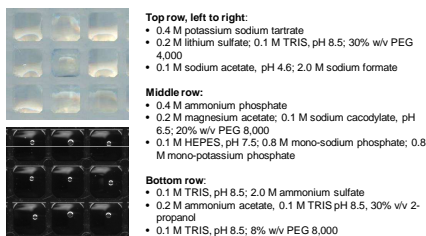


Figure 3: Acoustic transfer of nine protein crystallization fluids. 3 x 3 grid of source wells (top) shown with corresponding wells in destination plate (bottom). Each destination well is targeted with 20 droplets (2.5 nL nominal) that are shown coalesced into a single spot. Spot size and meniscus shapes may vary with fluid composition.

Experiment 1C: Protein Crystal Formation

A protein crystal was grown *in situ* by adding 25 nL of protein solution (Genentech proprietary protein) to 25 nL protein crystal standard and incubated overnight at room temperature. Both the protein and the crystallization fluid were transferred with an Echo liquid handler.



Figure 4: A schematic diagram of an Intelli-Plate well with 3 shelf positions and 1 reservoir (Art Robbins Industries, Sunnyvale, CA) and a cluster of protein crystals grown from an Echo liquid handler transfer (photo courtesy of Seth Harris, Genentech, South San Francisco, CA).

EXPERIMENT 2:

Formulate microvolumes of intermediate solutions through spot-on-spot placement.

Methods

Solutions of food coloring in anhydrous DMSO were prepared. 40 μ L of both blue and yellow-colored DMSO were placed in wells of a 384-well Echo qualified polypropylene plate. Fluid was transferred from the wells to the surface of a heat-sealed 384-well plate (white surface). The total volume transferred to each position was kept constant at 35 nL fluid (fourteen 2.5 nL droplets). The ratio of the differently colored DMSO solutions was changed for each transfer.

Results

The droplets of dye-spiked DMSO were transferred from source to destination. The droplets were transferred with spot-on-spot precision. All droplets coalesced into a single pool. Colors appeared homogeneous immediately after transfer suggesting that mixing was rapid and thorough. There was no sign of daughter drops indicating that there were no satellites and that transferred samples did not splash upon impact.



Figure 5: From left to right: 35 nL blue dye in DMSO; 30 nL blue, 5 nL yellow; 25 nL blue, 10 nL yellow; 20 nL blue, 15 nL yellow; 15 nL blue, 20 nL yellow; 10 nL blue, 25 nL yellow; 5 nL blue, 30 nL yellow; 35 nL yellow. Each spot is < 500 μ m in diameter.

EXPERIMENT 3:

Transfer of microcrystals

Methods

Hen egg white lysozyme (Sigma-Aldrich) crystals were grown by combining 200 μ L of lysozyme solution (200 mg/mL in 0.1 M sodium acetate, pH 4.5) with 200 μ L of crystallizing solution (0.1 M sodium acetate, pH 4.5, 1.0 M sodium chloride, and 50% glycerol) in a microcentrifuge tube and placing the mixture on a rocker overnight. The resulting lysozyme microcrystal slurries were acoustically transferred, in aliquots of one to four 2.5 nL droplets, to a MiTeGen 400/25 micromesh. Immediately after capture the mesh was plunged into a liquid nitrogen bath. The micromesh was held by a custom support.

Results

Three droplets containing microcrystals slurries of lysozyme were acoustically transferred to a single mesh. These crystals were later analyzed successfully on the beamline at Brookhaven National Laboratories⁵. Crystal structures were determined to better than 2 Å resolution from merged data sets.

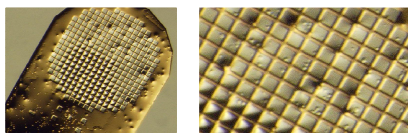


Figure 6: Left: MiTeGen mesh 400/25 to which three 2.5 nL droplets containing lysozyme microcrystals have been transferred. Right: Close-up of same screen. Small crystals measure less than 5 μ m along longest axis.

EXPERIMENT 4:

Non-invasive monitoring of protein crystallization fluids

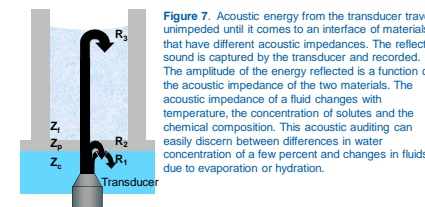
Methods

Pulses of acoustic energy were directed into microwells containing protein crystallization fluids. At any interface between materials where the acoustic impedance is not identical, some of the energy will be reflected back towards the transducer (i.e., echoed). The rest of the energy will be transmitted to the next material. By measuring the amount of energy reflected it is possible to monitor small changes in the acoustic impedance of the fluid. The amplitude of the energy reflected back to the transducer (R_2) is a function of the acoustic impedance of the material through which the acoustic pulse has passed and the acoustic impedance of the material that the pulse is entering. The acoustic impedance is affected by the temperature of the materials through which the energy flows.

In the case illustrated in Figure 7, the acoustic impedance of the coupling fluid, Z_0 , and the impedance of the plastic plate, Z_1 , are known. By measuring the reflected energy, R_1 , it is possible to adjust for temperature effects. By measuring the energy reflected at the interface between the plastic plate and the fluid in the well, it is possible to determine the acoustic impedance of the fluid, Z_2 . The acoustic impedance of the fluid is affected by the chemical composition of the fluid as well as by its concentration. As fluid evaporates, the concentration changes leading directly to a change in Z_2 . In the case of hygroscopic fluids such as DMSO, the Echo system has already shown that it can easily measure small changes in the amount of absorbed water⁶.

Results

Acoustic impedance of protein crystallization fluids were measured immediately upon transfer to a 384-well plate. The impedance was subsequently measured three weeks later. At that time 9 out of 98 wells showed changes in acoustic impedance that were more than 1 standard deviation from the mean change with one sample showing a change of more than two standard deviations in change. The impact of these changes still needs to be correlated with changes in crystallization efficiency.



EXPERIMENT 5:

Transfer lipidic cubic phase components to facilitate the self-assembly of nanoliter-scale environments for membrane protein crystallization

Methods

50 nL of deionized (MilliQ) water was transferred to a destination plate followed immediately by 225 nL monolein in methanol (2:1 by weight) transferred “spot-on-spot.” After 3 minutes (to allow methanol to evaporate) the process was repeated four more times with a 3 minute delay time between cycles. After the fifth round of transfers, the plate was transferred to an incubator at 30°C.

Results

A non-birefringent disk was observed at the bottom of the well after 3 hours. Manual probing of the disk revealed the highly viscous nature of the resulting material, supporting the hypothesis that the material has formed a lipidic cubic phase. Functional tests of this method of lipidic cubic phase will be performed in the near future.



Figure 8: Putative LCP forms spontaneously.

SUMMARY

These experiments show that acoustic droplet ejection is a viable tool for application in protein crystallography. We were able to transfer protein solutions and crystallization fluids. The high precision and accuracy for both volume transfer and positional placement provides a simple way to miniaturize crystallization experiments while broadening the number of different fluid compositions tested. The Echo platform was tested in five areas for utility in protein crystallization:

1. Acoustic droplet ejection with an Echo liquid handler can transfer the various fluids used in protein crystallization as it automatically adjusts for viscosity and surface tension. Over 1,400 different protein crystallization fluids have been transferred to date.
2. The positional placement of the Echo liquid handler allows the spot-on-spot placement application of fluid droplets to form intermediate concentrations of crystallization fluids.
3. Acoustic transfer of microcrystals have been shown to transfer, without damage, crystals suitable for X-ray diffraction studies.
4. It is possible to measure changes in protein crystallization solutions non-invasively with the analytical functions that are included with an Echo liquid handler.
5. It is possible to transfer all the components needed to form a lipidic cubic phase acoustically. The LCP appears to spontaneously form when nanoliter volumes are transferred.

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