**Automated Gradient Equilibration of Macromolecular Crystals to New Solution Conditions**

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**Abstract**

We describe a device and method for changing the ambient solution of a macromolecular crystal. The approach is gentle, automated, inexpensive and open source. Examples are given of equilibration of three different crystals to new solutions with exchange times ranging from 15-50 minutes. The system offers a more systematic and labor-saving workflow than current practice both for performing diffraction analysis of macromolecular crystals and for investigating the response of macromolecular crystals to changes in solution composition.

**Introduction**

Macromolecular crystals are commonly grown from a wide range of solution conditions, including aqueous solutions of salts, PEGs and alcohols. During crystal growth, the solution becomes integral, permeating the crystal in nanometer-sized pores. Recognizing that the solution is important for maintaining crystalline order was a key development in the progression of X-ray diffraction analysis to be a viable tool[Awk] for structure determination of macromolecules {Bernal, 1934 #1088}.

However, often the crystal growth solution must be changed due to the overall experimental objectives or the requirements of downstream experimental details. Some examples include changing the pH or salt conditions for functional reasons, adding a cryoprotective agent for low temperature data collection, and adding an inhibitor to the solution of an enzyme crystal.

Changing the solution can damage the crystal in a variety of ways. The crystal may simply dissolve if the macromolecule is more soluble in the new solution, or the new condition may trigger an alternative packing arrangement that cracks the crystal [REF]. Alternatively, the crystals may remain insoluble without significant repacking in the new solution but suffer cracking from the rate of change of the solution conditions[REF]. Here we will focus on reducing kinetic effects – that is, eliminating crystal damage that results from the rate of change of the crystal solution.

Crystal solutions are commonly changed with a variety of strategies. The simplest is to move the crystal directly into the new solution. In one early example, crystals were transferred from growth solutions containing high salt into organic solvents in a single step using a Pasteur pipet {Petsko, 1975 #1665}. However, direct single step transfer is often unsuccessful, yielding cracked crystals unsuitable for diffraction analysis. In these cases, it often helps to reduce concentration gradients experienced by the crystals, which has been approached in several ways.

First, direct transfer can still be used but in multiple steps using solutions of intermediate concentrations. In this case even very short soaks at the intermediate concentrations can be effective. A second approach is to leave the crystal in place, and change the crystal solution via pipetting. This is less damaging than direct transfer because the crystal experiences smaller concentration gradients {Garman, 2006 #170}, and repeated additions can yield a very gentle overall gradient {Garman, 1999 #164}. Crystal manipulations can also be performed in a humid environment, which reduces the rate evaporation from small droplets, and limits crystal drying during transfer between drops {Farley, 2014 #1159}. Despite the effectiveness of these approaches, they involve a fair amount of handling with multiple pipettings and/or transfers which create multiple opportunities to damage the crystals.

Vapor diffusion has been employed in at least two different ways. First, crystals can be pre-equilibrated against the new solution via vapor diffusion, making subsequent liquid transfer more successful {Garman, 2006 #170}. Second, vapor diffusion can directly deliver new solution components, as was shown for quickly delivering volatile cryoprotective agents to loop-mounted crystals. {Farley, 2014 #1158}. Additionally, liquid droplets have been used to deliver ligands to crystals and change crystal solutions. This has been done via acoustic droplet ejection {Collins, 2017 #1676}, and aerosolization {Ross, 2021 #1668} {Juers, 2022 #1677}.

Other researchers have shown crystals grown in gels are more robust to soaking induced damage {Sauter, 2009 #1705} {Sugiyama, 2012 #1125}. No doubt there are other inventive approaches that researchers have used over the years to successfully equilibrate their crystals to new solutions that have gone unreported.

Here, we focus on the traditional approach of gradually changing the crystal solution over time via repeated additions of a new solution to a small reservoir containing the crystals. Rather than manual pipetting however, we employ an open-source syringe pumping system capable of extremely low flow rates. The method is gentle, automated, inexpensive and open source.

**Materials and Methods**

*Chemicals and crystals.* All proteins, crystallization reagents and other molecules were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Crystals were grown using hanging drop vapor diffusion in 24 well plates at 284-298 K and used within a few weeks of grown. Tetragonal lysozyme (#L6876) well: 20 mM NaOAc 4.5, 3-8% w/v NaCl; protein: 80 mg/mL in 20 mM NaOAc 4.5 ([Forsythe et al., 1999](#_ENREF_11)). Thermolysin (#P1512) well: 2 M AmSO4; protein: 100 mg/mL in 45% v/v DMSO ([Hausrath and Matthews, 2002](#_ENREF_15)). Alpha lactalbumin (#L5385) well: 50 mM KH2PO4/15-20% PEG 8000; protein 30-50 mg/mL in water (Mueller-Dieckmann *et al.*, 2007).

*Device fabrication*

The auto serial dilution system is based on an open-source syringe pumping system {Booeshaghi, 2019 #1463}, and was fabricated using in house facilities. The hardware and software are all open-source, and are available with assembly instructions on GitHub {Juers, 2024 #1706}. Syringe pumping parts were printed in house with a Makerbot Replicator Gen 5 FDM 3D printer and PLA filament. The framework for holding the crystal pot and fluid tubes was designed using OpenSCAD, and also 3D printed in house. The syringe pumping software was modified with several options useful for crystal equilibration problems, including adding a menu to allow for user defined gradient flow (with Qt Designer).

*Crystal equilibration*

First, the syringe pumping system is prepared with the target solution in a one syringe and another syringe that is empty. Both syringes [Maker] are prepared with a needle [Maker] and small length of tubing [Maker]. The appropriate gradient parameters are entered into the software. Once the system is set up, the crystal is prepared.

Using a cryoloop or a pipet, the crystal is transferred from the growth drop into a small volume of solution (the well solution or mother liquor compatible with the crystal) in a PCR tube cap [Maker]. We normally use 40 µL. The reservoir is placed on a coverslip, which is placed in the jig on the microscope. The outflow and inflow tubes are position in the PCR tube cap, and the camera is focused on the desired location in the reservoir (typically the crystal). The gradient is then run. After the gradient is run, the crystal can be mounted for diffraction analysis or stored for later use.

**Results and Discussion**

Fig 1 shows a schematic of the strategy and its physical realization in the current system. Fluid is simultaneously removed (outflow) and added (inflow) at user defined rates, yielding gradual solution exchange. The arrangement of the inflow tube and the crystal can be adjusted depending on the relative density of the solutions. If, for example, the incoming solution has greater density than the existing solution, we place the crystal offset to the side from the inflow tube so the higher concentration solution doesn’t simply fall directly on top of the crystal. This measure isn’t always required, but can be helpful for particularly sensitive samples.

Fluid flow is controlled with an open-source syringe pumping system (Fig 2), includes three syringe pumps consisting of stepper motors mounted in a framework. The rotation of the stepper motors is coupled to translation of a syringe plunger. Because stepper motors are very precise the average flow rates of the syringe pumps can be very small – as low as xxx. The stepper motors are controlled with an Arduino microcontroller, through an open-source Python program running on a desktop or laptop. Within the software, the user sets up a gradient flow system, setting the flow rates and rate of fluid exchange (Fig 2b). As shown the system is controlled with a dedicated Raspberry pi computer.

Fig. 3 shows some example crystal equilibrations, comparing single-step transfers to exchanges done with the auto serial dilution system. In each case the crystals sit in approximately 40 µL of solution, which gets exchanged over time.

Panel A shows equilibration of crystals of alpha-lactalbumin to a glycerol based cryosolution. The crystal started in well solution ( ), which was gradually exchanged with 20% PEG8K, 50 mM salt, 25% glycerol. A 50-minute linear gradient yields an intact crystal, while the single step transfer causes substantial cracking.

Panel B shows equilibration of the hexagonal crystal form of thermolysin to pure water. Thermolysin has remarkably low solubility at low salt to the point that crystals are stable in small volumes of deionized water. Here the crystals started in 2 M AmSO4, which is similar to the well solution, and were equilibrated to deionized water with a linear gradient over 15 minutes.

Panel C shows equilibration of crystals of the tetragonal crystal form of lysozyme to lower salt conditions. Lysozyme is a model system for investigations of the response of crystals to changes in solution conditions. {Lopez-Jaramillo, 2002 #1133} Here the starting solution was the well solution of 40 mM NaOAc, pH 4.2 [Check], 8% NaCl, and the final solution was 40 mM NaOAc, 3% NaCl. Direct transfer produces roughly parallel cracks, due to solvent movement into the crystal under hypodonic conditions {Lopez-Jaramillo, 2002 #1133}. However, a 40-minute linear gradient yields intact crystals.

In each case described, the crystals can be equilibrated to the new solution via direct transfer in smaller concentration steps or by repeated manual pipetting. However, the repeated transfers required constant attention, and present more opportunity for something to go wrong and for the crystals to be damaged. Here, there is only one transfer needed to equilibrate to the new solution, and once the gradient is started, the crystal(s) can be left unattended and the user’s attention can be directed elsewhere while the crystals are equilibrating. Furthermore, the solution gradient may be as shallow as required to yield an intact crystal. Here we found that equilibration times of < 60 minutes were adequate. However, one could imagine longer gradients being employed. It is certainly possible that crystal order and diffraction quality could be compromised even though visually the crystal seems intact. This system creates the opportunity for more systematic investigations of the effects of equilibration rate on crystal order. We note, however, that as the equilibration time increases, measures to reduce evaporation may be needed, using for example a humid flow device {Farley, 2014 #1159}.

Here we used a reservoir volume of 40 µL. Although small, this volume is still large enough that in some cases dissolution of the crystals when placed in the reservoir yields some surface degradation. This can be prevented by adding a low concentration of the protein to the initial and final solutions.

**Conclusion**

We have assembled a system to facilitate crystal solution exchange with minimal handling. Inexpensive and straightforward to build using open-source components, the system should be a useful tool in the workflow of structural biology labs. Additionally, the tool may facilitate more systematic studies of the responses of crystals to solution concentration gradients.