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High throughput screening using acoustic droplet ejection to combine protein crystals and chemical libraries on crystallization plates at high density

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ABSTRACT

We describe a high throughput method for screening up to 1728 distinct chemicals with protein crystals on a single microplate. Acoustic droplet ejection (ADE) was used to co-position 2.5 nL of protein, precipitant, and chemicals on a MiTeGen *in situ-1* crystallization plate™ for screening by co-crystallization or soaking. ADE-transferred droplets follow a precise trajectory which allows all components to be transferred through small apertures in the microplate lid. The apertures were large enough for 2.5 nL droplets to pass through them, but small enough so that they did not disrupt the internal environment created by the mother liquor. Using this system, thermolysin and trypsin crystals were efficiently screened for binding to a heavy-metal mini-library. Fluorescence and X-ray diffraction were used to confirm that each chemical in the heavy-metal library was correctly paired with the intended protein crystal. A fragment mini-library was screened to observe two known lysozyme ligands using both co-crystallization and soaking. A similar approach was used to identify multiple, novel thaumatin binding sites for ascorbic acid. This technology pushes towards a faster, automated, and more flexible strategy for high throughput screening of chemical libraries (such as fragment libraries) using as little as 2.5 nL of each component.

1. Introduction

High throughput screening using X-ray crystallography is a powerful tool for applications such as fragment library screening (for structure-based drug discovery) and additive library screening (for improving crystal quality) (Spurlino, 2011). For structure-based drug discovery projects (Blundell et al., 2002) the screened library may consist of individual fragments (Chilingaryan et al., 2012) or pooled fragments (Nicholls et al., 2010). For crystal quality improvement projects the screened library may contain additives such as protic ionic liquids (Kennedy et al., 2011). The chemicals being screened can be added either before crystal formation (co-crystallization) or after the crystals have grown (soaking). With either method, the objective is to screen a library which may consist of several thousand chemicals using a minimum amount of purified protein, screened chemicals, and other consumables.

Acoustic droplet ejection (ADE) has a demonstrated utility for growing protein crystals (Villasenor et al., 2012), improving the quality of protein crystals (Villasenor et al., 2010), mounting protein crystals onto data collection media (Soares et al., 2011; Roessler et al., 2013), and for high throughput screening of protein crystals (Yin et al., 2014). ADE uses a sound pulse (Fig. 1) to propel a liquid or suspended solid out of a source location, through a short air column, and onto an arbitrary destination (Ellson et al., 2003; volume accuracy 5%, trajectory precision 1.3°). The high trajectory precision enables "drop on drop" combination of distinct components from different source wells onto the same destination

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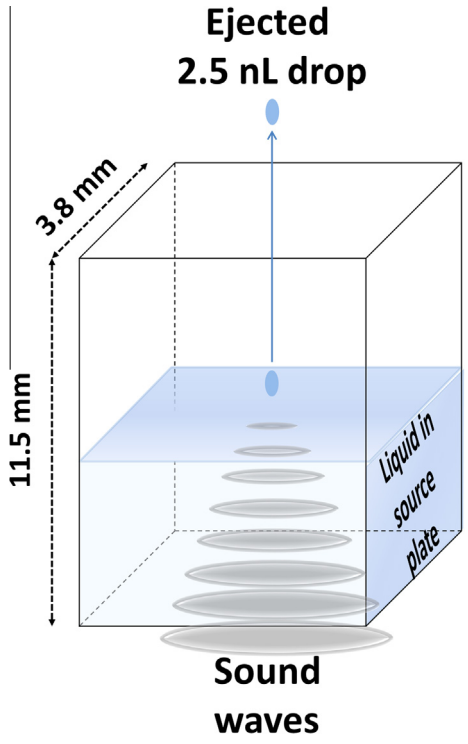

Fig. 1. Acoustic droplet ejection. ADE uses sound energy to transfer variable micro-droplets (e.g. nanoliter or picoliter) of solution (protein, precipitant, chemicals, etc.) from a well in a source plate through a short air column (~1 cm) to data collection media. Sound wave energy from the transducer is channeled to the focal point (i.e. ejection zone) displacing the surface where a controlled ejection occurs. Droplet size is governed by the wavelength of the sound emitted. In this work an Echo 550 was used to combine proteins, precipitants, and chemicals for co-crystallization and soaking experiments in 1728 distinct locations on a MiTeGen *in situ* crystallization plate. The Echo 550 does not use frequency changes to transfer different volumes. Instead, it uses a fixed frequency sound pulse to transfer each component in 2.5 nL increments.

location. The trajectory precision is sustained across a wide variety of commercially available crystallization conditions and cryo-protectants (Cuttitta et al., 2015). The Echo 550 acoustic liquid handler used in this study (Labcyte Inc., Sunnyvale, CA) transfers liquids using increments of 2.5 nL (ejection velocity ~1 m/s).

Villasenor and co-authors have suggested that acoustic methods might be used for structure-based drug discovery by co-crystallizing protein and fragments using a shared reservoir on a conventional crystallization plate (Villasenor et al., 2012, 899¶6). Note that this strategy employs the same precipitant to drive crystallization in all 96 wells of the crystallization microplate. Acoustic methods are an attractive choice for micro-crystallization for several reasons. ADE is an automated technique that does not rely on operator skill. It is physically gentle with no tips or tubes that may leach chemicals, cause cross-contamination between specimens (McDonald et al., 2008), or damage crystals. Transfers have high accuracy even at very low volume (2.5 nL) with zero per transfer "lost volume" since there are no tips or tubes that liquids can adhere to. The inaccessible "dead volume" at the bottom of each well is very small (4 μL; Harris et al., 2008) and can be reduced even further (Cuttitta et al., 2015). Specimen transfer is fast (500 mounts per second between fixed locations; 2.33 ± 0.04 mounts per second to multiple destinations, data not shown), which reduces specimen preparation time. Our system for room temperature fragment library screening is keyboard controlled and remote compatible, and can be readily mastered by new users.

As the working volume for crystallization drops below ~50 nL, dehydration becomes the foremost challenge. Our group made a previous attempt to develop the on-plate high throughput screening technology proposed by Villasenor et al. in 2012 without success (and we know of other similar attempts by other groups from personal communications). We tried several strategies for mitigating the impact of dehydration, such as working at 4 °C, parsing big jobs into small pieces, or simply working faster. None of these mitigation strategies were sufficient when dealing with 2.5 nL working volumes. To reduce dehydration we transferred all protein, precipitant and fragment components through small apertures in a plate lid that covered the MiTeGen *in situ-1* crystallization plate™ (Zipper et al., 2014). The MiTeGen plate was allowed to equilibrate with the precipitant before the crystallization fluids were ejected onto it (Fig. 2). A separately designed custom plate lid was used to cover the source plate (that contained the mini-libraries) to prevent the dimethyl sulfoxide (DMSO) that was solvating the chemicals from swelling with incorporated water when exposed to atmospheric humidity.

Here we describe a high throughput technique for screening protein crystals against a chemical library, using ADE to prepare co-crystallization or soaking experiments on MiTeGen crystallization microplates (Fig. 3). A critical advantage of ADE is that it can deposit solutions at their intended destination with a very high positional accuracy. The capability to combine protein, precipitant, and screened chemicals at 1728 distinct locations on a MiTeGen crystallization microplate is one advantage of this positional accuracy that we have already mentioned. Since the precise location of each of these 1728 experiments is known, there is also the opportunity to automate the data acquisition process by programming the plate-handling system (such as the G-rob system used in this work) to rapidly move between the 1728 known specimen locations. *In situ* data can then be obtained under computer control so that high throughput screening can occur without operator assistance. The speed and automation of this data acquisition approach could facilitate structure-based, high throughput fragment screening without grouping chemicals into cocktails. This mitigates the harmful effects of a high aggregate fragment concentration on protein stability and crystallization (Boyd and de Kloe, 2010; Baurin et al., 2004), prevents inter-fragment interactions (Drinkwater et al., 2010; Nair et al., 2012), and avoids the need to de-convolute the fragments in each cocktail after a hit (Nicholls et al., 2010).

2. Methods

We used two techniques for high throughput screening of proteins against chemical libraries, using X-ray crystallography as the primary screening tool:

Co-crystallization of protein with a library of chemicals: DMSO-solvated libraries were co-crystallized with protein and screened *in situ* (Fig. 4A). The library chemicals were acoustically deposited on the crystallization plate before any other components were added, and the DMSO was allowed to evaporate (leaving the dry residue of the chemical)[2]. The reservoir was then filled with mother liquor and the crystallization plate was sealed with a custom fabricated plate lid (Fig. 2) (Zipper et al., 2014). Once the

[2] DMSO is widely used as a solvent for chemical libraries because it is polar (with properties similar to water) but it is aprotic (which extends the useful life of the chemicals in the library). However, DMSO is a protein denaturant that is poorly tolerated by many crystals (Arakawa et al., 2007). It is often desirable to remove the DMSO immediately before each chemical is combined with proteins. In our experiments DMSO could be removed by allowing the DMSO solvent to evaporate before the protein and precipitant were added (on top of the dry residue containing the chemical). Of course, this approach is only practical when screening non-volatile chemicals.