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A new view on crystal harvesting

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X-ray crystallography typically requires the mounting of crystals, which can make the sample difficult to manipulate when it is small and the microscope objective is close to the crystallization plate. By simply moving the objective to the bottom of a clear crystallization plate (inverting the normal view), crystals were able to be manipulated and harvested from wells having a 0.9 mm diameter and 5.0 mm depth. The mounting system enabled the structural solution of the 187 amino acid N-terminal domain of *Saccharomyces cerevisiae* glutaminyl-tRNA synthetase from crystals that appeared during high-throughput screening but proved recalcitrant to scale-up and optimization. While not a general mounting solution, the simple expedient of removing the objective lens from the area where manipulation and harvesting occur greatly facilitates the manual, or even automated, process.

1. Introduction

Typically, for successful X-ray crystallographic data collection a crystal is placed within the X-ray beam and then rotated through an appropriate angle to record a complete data set. Historically this was accomplished by harvesting the crystal from the conditions it was grown in and then sealing it within a glass capillary to prevent its dehydration. In the majority of cases today, a polymer loop or tip is used to harvest the crystal, and then the crystal is preserved in a cryogenic gas stream. While other approaches have been developed, including *in situ* methods (McPherson, 2000) and even jets of nanocrystals coupled with intense X-ray laser sources (Chapman *et al.*, 2011), harvesting and mounting of crystals will probably remain the status quo for many years to come. While harvesting and mounting requires some skill and dexterity, in most cases it is achievable, and it is a standard practice within the field.

Obtaining the initial crystals is a largely empirical process. At the Hauptman–Woodward Medical Research Institute we operate a high-throughput crystallization screening laboratory that requires 450 µl of sample to assay against 1536 different chemical conditions known to promote crystallization. The screening process is described in detail elsewhere (Luft *et al.*, 2003, 2011). The crystallization method used is the microbatch-under-oil method (Chayen *et al.*, 1992). Because of the large number of experiments and the small volume used, 200 nl of macromolecular solution and 200 nl of potential crystallizing cocktail (covered by 5 µl of mineral oil), the experimental wells used are narrow, having a diameter of 0.9 mm at the base and a well depth of 5.0 mm. Typically, when a condition indicates the potential to produce a crystal suitable for diffraction studies, the experiment is scaled up into a larger-format plate to enable crystal extraction. This may not always be possible or successful; in some cases the amount of macromolecule may be limited, there may be time constraints and in a few cases scale-up may be unsuccessful.

In this paper we report on a case where crystallization screening of a construct of the N-terminal domain (residues 1–187) of *Saccharomyces cerevisiae* glutaminyl-tRNA synthetase was not reproducible during scale-up to larger volumes. This was an in-house target of considerable interest. To circumvent this issue, we developed a method to extract, mount and determine the structure of crystals harvested directly from the microassay plates used for initial crystallization trials. The key step was the ability to directly observe the crystal during extraction from the plate, unobstructed by the method of harvesting – a problem that had previously impeded attempts to remove crystals from the small and narrow wells of the microassay plate. We describe the methodology, which is generally applicable to other crystallization methods, and instrumentation we developed to realize the eventual structural knowledge.

2. Experimental

2.1. The sample and crystallization plate

Our protein sample was the 187 amino acid N-terminal domain (NTD) of *Saccharomyces cerevisiae* glutaminyl-tRNA synthetase (Gln4). The experimental details of the expression, purification, crystallization and resulting structure have been published elsewhere (Grant *et al.*, 2012). Crystallization conditions were identified using the high-throughput screening approach described above, with crystals appearing after six-week's incubation at 295 K (22°C). Crystals were observed in two chemical cocktails. Each protein solution consisted of 0.2 µl of 8.9 mg ml^{-1} protein in 100 m*M* NaCl, 5%(*v*/*v*) glycerol, 2 m*M* dithiothreitol, 0.025%(*w*/*v*) NaN_3, 20 m*M* HEPES buffer pH 7.5 and 0.2 µl of precipitant solution. The respective cocktail solutions were (*a*) 100 m*M* KCl, 100 m*M* Tris pH 8.0 and 20%(*w*/*v*) PEG 4000 and (*b*) 100 m*M* LiCl Tris pH 8.2 and 20%(*w*/*v*) PEG 4000. Attempts to reproduce the crystals in a larger-volume microbatch experiment and using vapour-diffusion techniques failed.

The microassay plate used by the high-throughput crystallization screening laboratory is a Greiner Bio-One 1536-well Imp@ct low-birefringence plate with a base of transparent polyolefin. Each of the 1536 wells in this plate has a depth of 5.0 mm with a flat bottom. The well opening is a square, with a diameter of 1.7 mm, tapering to a circular bottom with a diameter of 0.9 mm. Combined, this depth and diameter make it practically impossible to simultaneously insert a loop or capillary and visualize the extraction process from the same

side of the plate. The *in situ* observation and harvesting system circumvents this problem by viewing and extracting from opposite sides of the plate.

The transparent crystallization plate and thin bottom, ∼1.0 mm, makes it possible to observe experiments contained in the wells and any crystals that have formed by viewing through the bottom of the plate. When the plate is inverted, surface tension holds the oil and experiment drops in place. The crystals remain stationary within the wells. While the plates are stored upright, this property is exploited during the crystallization screening, as the plates are imaged from the top in the inverted position (Luft *et al.*, 2003, 2011). With a transparent material we can deconvolute the observation and extraction such that the observation is carried out through the solid base of the plate and the extraction through the open top. A simple observation and harvesting system was constructed to accomplish this by aspiration of the solution from the well (Fig. 1). The system consisted of a CCD camera and a telescopic lens, to view the plate from below, an *XY* translation stage that was open in the centre (for illumination and well visualization) to hold the crystallization plate, and an *XYZ* translation stage, holding a brass tube guide containing Tygon tubing connected at one end to a Hamilton syringe with the other end (the harvesting end) positioned vertically above the crystallization plate. The system was constructed from available hardware in the laboratory and was not optimized for size or elegance.

For optimal aspiration of multiple crystal sizes and to minimize contamination between wells containing different crystallization conditions, we used a small polyester tube (Advanced Polymers Inc, Salem, NH, USA) at the harvesting end of the tubing. This tube was glued to a shortened gel-loading pipette tip to make the harvesting assembly and inserted into the Tygon tubing (Fig. 2). The low-cost polyester harvesting tube for this assembly is available with a wall thickness as small as 2.5 µm and inner diameters ranging from 0.1 to >1.0 mm to appropriately match crystal size. Illumination is possible from both below and above and was optimized in each case to obtain the best view of the crystal.

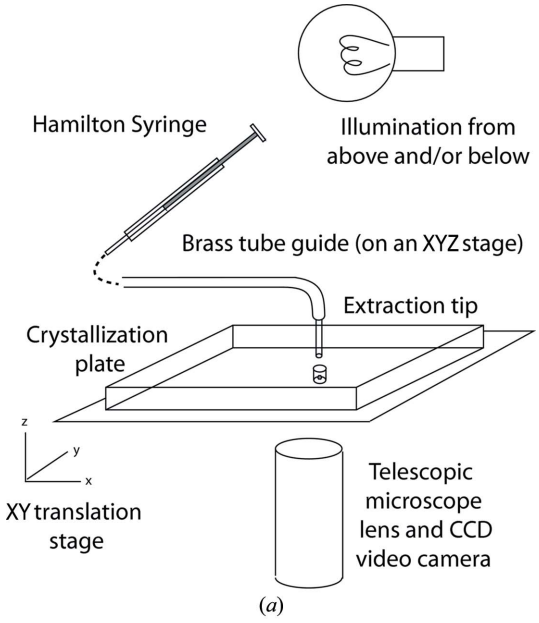

Figure 1
Schematic of the system (*a*) and a photograph (*b*) of the actual system. Note that the Hamilton syringe is not visible in (*b*) and that two sources of illumination are used, reflected illumination from the top and illumination from the bottom. The *XY* translation stages are not labelled. The brass tube sits on one while the crystallization plate sits on another.

2.2. Mounting, cryocooling and data collection

To prepare for crystal extraction, the crystallization plate is placed on an *XY* translation stage and the well of interest containing the crystal(s) is positioned vertically above the fixed telescopic lens. Guided by the output of the imaging system, the *XY* translation stage is manoeuvred to precisely align the well with the centre of the image and the focus is adjusted to ensure the crystal(s) are within the depth

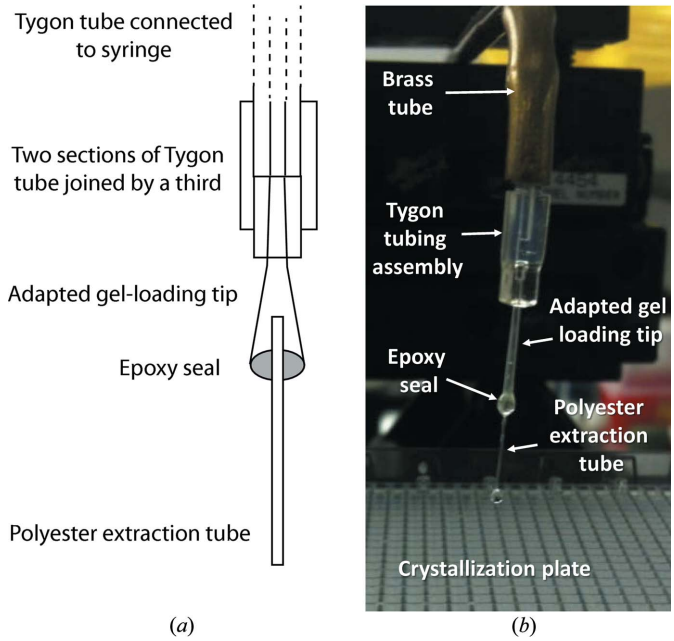

Figure 2
(*a*) A close-up of the harvesting assembly (adapted gel-loading tip and polyester extraction tube) part of the extraction tip used for extraction and mounting of the crystal. (*b*) A drop of mineral oil is extruded from the extraction tube.

of field. An appropriate harvesting assembly is inserted into the Tygon tube (Fig. 2*a*). The syringe, preloaded with oil, is then used to load this tip with oil so that no compressible air gaps are present within the system. This incompressible fluid is critical for precise nanovolume liquid transfers. The harvesting tube is then positioned over the well of interest using the *XYZ* translation stage that holds the harvesting assembly. At higher magnifications, a short depth of field impedes the ability to see the extraction tube directly in the video output until is it lowered into the well; thus the initial positioning is guided by eye. The harvesting tube is slowly lowered into the well over the targeted crystal and aligned with precision as it enters the field of view of the imaging system. In the case of crystals spanning a large portion of the well, or where a cluster of crystals is present, the tube may be lowered to the base of the crystallization well, 'cutting' the cluster and separating it into individual crystals. The tube is centred on crystals of interest and a slight and gentle aspiration with the syringe is used to remove the crystal, mother liquor and, depending on the aspiration, some mineral oil from the well. Matching the diameter of the tube to the crystal ensures that a minimum volume of liquid is used and that the other crystals in the same well are not disrupted and can be sequentially harvested by the same method. The construction of the harvesting assembly (the polyester extraction tube and adapted gel-loading tip) is designed such that it can be rapidly exchanged, for example, for different crystal sizes or wells. The harvesting tube is strong enough that it can be used to manipulate the crystals and dislodge them if required. Once the crystal is in the tube, the harvesting assembly is translated vertically upward and the crystallization plate removed. At this stage the crystal can be expelled directly into a cryoloop for mounting and cryocooling.

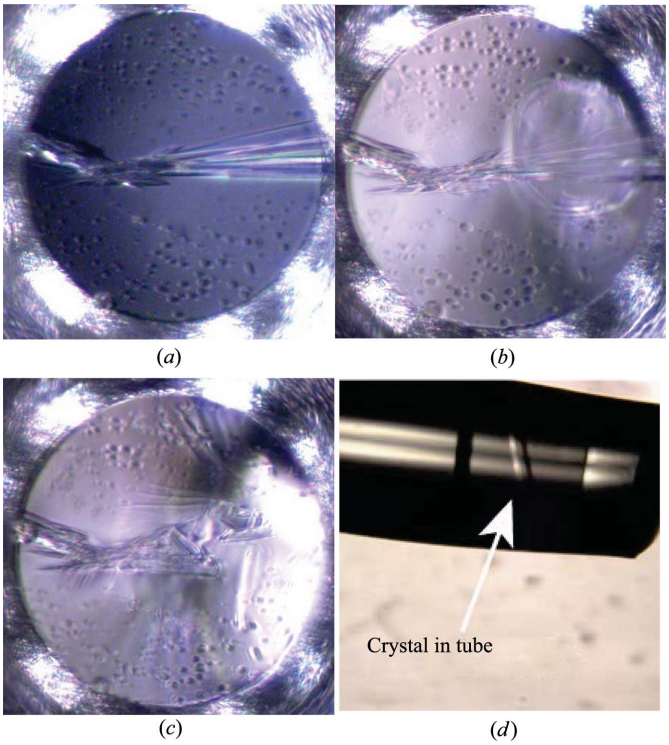

Figure 3
Time sequence monitoring the crystallization drop (*a*) before the polyester tube is inserted, (*b*) as it is inserted and positioned over the crystal, and (*c*) after it is removed and the crystal taken with it. In this case crystals spanned the entire well (0.9 mm in diameter) and the tube was used to separate the crystals and select only a single crystal to mount and subsequently cryocool. In (*d*), the crystal is shown in the polyester extraction tube (0.4 mm diameter) after extraction from the well. Note that the crystal and mother liquor are separated from the mineral oil above (already in the tubing) and below (extracted from the well) which does not mix.

3. Results

For the extraction of crystals from the screening plate of the Gln4 NTD, several large crystals were clustered in the well containing the KCl cocktail (Fig. 3*a*). The cluster was broken with the polyester harvesting tube and three crystals were extracted individually. Nine crystals were extracted from the well containing the LiCl cocktail. Fig. 3(*b*) shows the polyester tube positioned over the cluster, Fig. 3(*c*) the remains of the cluster once a single crystal had been extracted and Fig. 3(*d*) the crystal in the polyester mounting tube. The polyester tube used in this case is 0.4 mm in diameter and was chosen to be smaller than the diameter of the well and the length of the crystal cluster in order to cleave the cluster of crystals. We expelled mounting crystals from the harvesting tube directly into a nylon loop and then took the loop and crystal and plunged them into liquid nitrogen for cryopreservation.

The crystals were sent to Stanford Synchrotron Radiation Lightsource (SSRL) for remote data collection. Two out of the three crystals grown in the KCl cocktail diffracted. No visible diffraction was seen from the other KCl or LiCl samples. A complete data set was collected from the stronger diffracting of the two KCl-grown crystals, and this data set was successfully used to determine the structure shown in Fig. 4. Full details of the structure determination and analysis are given elsewhere (Grant *et al.*, 2012, 2013). Without the ability to harvest these crystals, given the failure to reproduce the crystallization results, the efforts expended would not have otherwise provided this structure.

4. Discussion and conclusion

In the majority of cases where a crystallization result is seen in our 1536-condition screening process, the crystal hit can be scaled up through microbatch-under-oil or vapour-diffusion methods in a different geometry plate. This scale-up process typically provides crystals that are larger and, more importantly, accessible to mounting. In the case presented here, despite extensive study, we could not reproduce the initial crystallization hit. To overcome this we constructed an *ad hoc* system to make use of the crystals we had. We

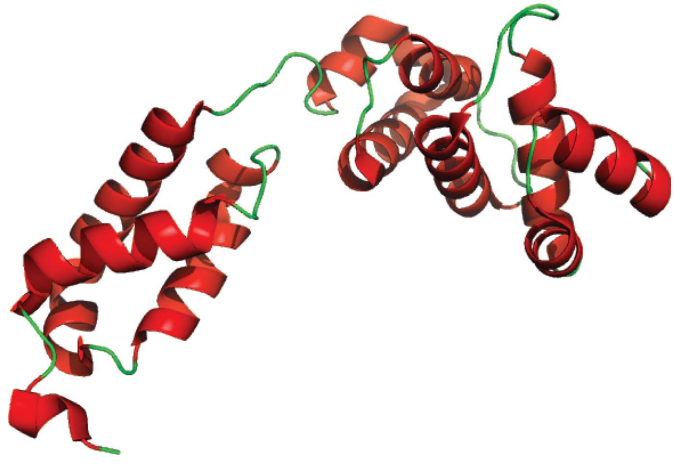

Figure 4
Structure of the N-terminal domain resulting from the harvested crystals (Grant *et al.*, 2012). Coordinates have been deposited as PDB ID 3tl4.

were forced to divide a cluster of crystals, cutting them and then harvesting 'single' crystals directly from the crystallization well. The ability to observe this process from the opposite side of the plate ensured that we could choose accurately where to cut the cluster and then select a single crystal from that cluster for harvesting. Our efforts were successful, demonstrating not only that the crystal could be extracted but that it could be manipulated, mounted and used for X-ray data collection. However, only two crystals out of the nine that were mounted provided diffraction data. The two crystals that diffracted were those mounted from the inside of the tube that did the cutting.

It is not clear if the lack of diffraction from seven of the nine crystals was due to the mounting procedure used (perhaps physical damage to the crystals), a failure in cryocooling, a general lack of short-range order within the crystals or some other cause. In terms of physical manipulation, the crystals certainly suffered from mechanical stress when they were 'cut'. In terms of cryocooling, no strong ice rings were seen, but this is not good evidence of an ideal cryoprotectant solution where small changes in conditions can have large effects on outcome (Mitchell & Garman, 1994).

While this example expelled the crystal from the harvesting assembly into a loop where it was directly cryocooled, the crystal can be easily expelled onto to a microscope slide (successful in other cases not described) and cryoprotectant solutions can be added if the initial solution was not suitable. Similarly, the extraction of the crystal into the polyester extraction tube could be used to mimic the mounting of crystals in capillaries (Bernal & Crowfoot, 1934). For example, the crystal can be left in the mounting tube where it was extracted, the tube cut at the epoxy seal, solution removed from around the crystal and the tube sealed at either end.

Other approaches have been developed to extract crystals reliably from constrained geometries or where manual dexterity is not sufficient: for example, robotic systems for harvesting then cryocooling reviewed by Deller & Rupp (2014). Alternatively, *in situ* studies and even the collection of data sets from the crystallization plate are possible (Jacquamet *et al.*, 2004).

Our approach had a poor success rate, but this was still good enough to provide the structure. We do not propose this instrumentation as a universal mechanism for extracting and mounting crystals, but note that a key feature for success, the observation of the crystal opposite from the harvesting side, is generally applicable to any case where the crystallization plate and solutions are optically transparent. While we have used this approach to overcome a constrained geometry in crystallization plates that are somewhat unique to our high-throughput crystallization screening process, the same approach can be easily adapted to a number of configurations, allowing direct observation of crystal manipulation and extraction. For example, the same method of observation greatly facilitates regular cryolooping by allowing easy unimpeded access to the drop. While we have used a video microscope system, the same approach could be carried out with a suitably modified inverted microscope. Regardless of the mechanics of crystal extraction, observation of the crystallization experiment from the opposite side of the extraction is a generally applicable technique. This mode of visualization will improve the access without impeding that visualization and therefore enhance the success of physically extracting crystals.

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