Transmission electron microscopy for the evaluation and optimization of crystal growth

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The crystallization of protein samples remains the most significant challenge in structure determination by X-ray crystallography. Here, the effectiveness of transmission electron microscopy (TEM) analysis to aid in the crystallization of biological macromolecules is demonstrated. It was found that the presence of well ordered lattices with higher order Bragg spots, revealed by Fourier analysis of TEM images, is a good predictor of diffraction-quality crystals. Moreover, the use of TEM allowed (i) comparison of lattice quality among crystals from different conditions in crystallization screens; (ii) the detection of crystal pathologies that could contribute to poor X-ray diffraction, including crystal lattice defects, anisotropic diffraction and crystal contamination by heavy protein aggregates and nanocrystal nuclei; (iii) the qualitative estimation of crystal solvent content to explore the effect of lattice dehydration on diffraction and (iv) the selection of high-quality crystal fragments for microseeding experiments to generate reproducibly larger sized crystals. Applications to X-ray free-electron laser (XFEL) and micro-electron diffraction (microED) experiments are also discussed.

1. Introduction

X-ray crystallography remains the most successful method for obtaining high-resolution structural information from biological macromolecules, and structures solved by this method comprise 89% of the current entries in the Protein Data Bank (<http://www.rcsb.org/>). Advances in molecular biology and biochemical techniques have allowed the expression and purification of human and pharmacologically relevant protein targets (Saïda *et al.*, 2006; Mokhonova *et al.*, 2005; Carpenter *et al.*, 2008). Similarly, innovations in synchrotron technology, including the development of micro-focused X-ray beams and advances in X-ray area detectors, as well as the development of user-friendly crystallography software, have significantly shortened crystal-to-structure time frames. In addition, substantial advances have been made in protein-modification methods to enhance the success of crystallizing biologically relevant protein targets (Blaber *et al.*, 1995; Cherezov *et al.*, 2007; Dupeux *et al.*, 2011; Hino *et al.*, 2012; Rasmussen *et al.*, 2011; Steyaert & Kobilka, 2011). However, while these

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Table 1

Protein purification and crystallographic conditions.

Protein†	Class	Crystallization condition‡	Average order of Bragg spots	Maximum resolution§ (Å)
Pol II $\Delta 4/7$ (Cramer <i>et al.</i> , 2001)	Soluble	0.4 M ammonium/sodium phosphate pH 6.5, 6–10% PEG 6000, 50 mM dioxane, 10 mM DTT	Third	3.2
Wild-type Pol II (Pullara <i>et al.</i> , 2013)	Complex	0.1 M HEPES pH 7.0, 0.3 M sodium malonate, 0.2 M ammonium acetate, 3–6% PEG 6000, 10 mM DTT	Third	3.5
Pol II-TFIIF-DNA (Pullara <i>et al.</i> , 2013)	Complex	0.1 M HEPES pH 7.0, 0.1 M sodium malonate, 8–12% PEG 4000, 10 mM DTT	Third First	3.8 (aniso) 5.0
Pol II-Spt4/5-DNA (Cohen <i>et al.</i> , 2014)	Complex	0.1 M MES pH 6.0, 0.2 M magnesium chloride, 10–14% PEG 2000, 10 mM DTT	Third Second	3.9 (aniso) 4.5
Pol II-TFIIB-DNA (Cohen <i>et al.</i> , 2014)	Complex	0.1 M HEPES pH 7.0, 30–35% Tacismate pH 7.0, 10 mM DTT	N/A	3.4
Pol II-GFP (Cohen <i>et al.</i> , 2014)	Complex	0.1 M HEPES pH 7.0, 0.8 M sodium citrate, 10 mM DTT	Third Second	3.6 (aniso) 4.3
Pol II-CD3 Δ (Cohen <i>et al.</i> , 2014)	Complex	0.1 M MES pH 6.0, 1.4 M sodium malonate, 10 mM DTT	Third Second	4.4 (aniso) 5.7
UNG2-DCAF1-DDB1-Vpr (Ahn <i>et al.</i> , 2010)	Complex	0.1 M Tris pH 7.5, 0.1 M sodium acetate, 15% PEG 4000	Third	3.7
DCAF1-DDB1-Vpr (Ahn <i>et al.</i> , 2010)	Complex	2.0 M sodium chloride, 12% PEG 6000	Third	3.6
PTHR (Pullara <i>et al.</i> , 2013)	Membrane	0.1 M sodium phosphate, 12% PEG 4000, 5 mM TCEP pH 6.2	Second	N/A
BRIL-PTHR¶	Membrane	0.1 M sodium citrate, 10% PEG 8000, 0.1 M calcium chloride, 10 mM DTT pH 5.8	First	N/A
2,3-HPCD (Botha <i>et al.</i> , 2015)	Soluble	0.1 M MES pH 5.8, 0.1 M calcium chloride, 12–14% PEG 6000	Fourth	1.55
Globin-X††	Soluble	0.2 M sodium malonate pH 7.0, 20% PEG 3350	First	<10
APOBEC (adapted from Byeon <i>et al.</i> , 2013)	Soluble	0.1 M MES pH 6.2, 1.5 M sodium citrate, 0.1 M HEPES pH 7.0, 0.2 M magnesium acetate, 10% PEG 4000	First Second	<10 6.3
Rtf1 (Dermody & Buratowski, 2010)	Soluble	0.1 M MES pH 6.0, 0.1 M magnesium chloride, 15% PEG 3350	First	7.3
dGTPase‡‡	Globular	1.6 M potassium/sodium phosphate pH 8.5	Fourth	2.8
<i>Sindbis virus</i>	Viral	n/a	First	<10

† Purification and complex-assembly protocols were performed as described previously for all proteins or complexes where a reference is provided. ‡ In all cases, crystallization was achieved using vapour diffusion with drop sizes between 1 and 2 μ l. § Maximum resolutions were visually estimated from diffraction patterns collected at synchrotron sources (APS, Chicago, Illinois, USA or SSRL, Stanford University, California, USA). ¶ Sf9 cells infected with BRIL-PTHR1 baculovirus and grown for 41 h were lysed by sonication in the presence of 300 nM LA-PTH, a long-acting analog of parathyroid hormone, and 2 mg ml⁻¹ iodoacetamide. Isolated membranes were extracted with 1% *n*-dodecyl β -D-maltoside (DDM) and 0.1% cholesteryl hemisuccinate. The extract was incubated with Ni Sepharose 6 Fast Flow (GE Healthcare) and eluted with 250 mM imidazole after exchanging the 1% DDM for 0.1% decyl maltose neopentyl glycol (DMNG). Pooled elution fractions were batch-bound with anti-FLAG M1 agarose-affinity gel (Sigma) and eluted with 10 mM EDTA and 0.1 mg ml⁻¹ FLAG peptide (Sigma) before being subjected to gel-filtration chromatography (Superose 6, GE Healthcare). †† Purified globin-X from zebrafish was a generous gift from the laboratory of Dr Mark Gladwin. ‡‡ Transformed *Escherichia coli* BL21(DE3) cells were grown at 37°C to an optical density of 0.6 at 600 nm and induced with 0.4 mM IPTG for 16 h at 18°C. Purification was first performed by Ni-NTA chromatography (GE Healthcare) followed by gel-filtration chromatography (HiLoad Superdex 200 16/60, GE Healthcare) equilibrated in 25 mM sodium phosphate pH 7.5, 150 mM NaCl, 1 mM DTT, 10% glycerol, 0.02% sodium azide.

improvements in the efficiency of the crystal-to-structure pipeline and protein-modification methods have led to successful three-dimensional structures of an increasing number of proteins, significant challenges still remain in crystallizing a variety of proteins of interest.

One such challenge is the inability to identify conditions that promote the spontaneous nucleation of macromolecules into a well ordered crystal lattice. Generally, this is considered to be the major limitation in protein crystallography experiments, especially when crystal growth is limited to nanometre-sized dimensions. Emerging techniques utilizing second-order nonlinear optical imaging of chiral crystals (SONICC; Kissick *et al.*, 2011), UV microscopy (Desbois *et al.*, 2013) and negative-stain TEM (Calero *et al.*, 2014; Stevenson, Makhov *et al.*, 2014) have shown that protein nanocrystals are ubiquitously present in granular aggregates of crystallization drops, which would usually be overlooked by the experimenter. Using TEM, we showed that on many occasions such nanocrystals contained well ordered lattices (Stevenson, Makhov *et al.*, 2014), which may represent an ideal material for novel injector-based serial femtosecond crystallography (SFX) experiments at X-ray free-electron laser (XFEL) sources or micro-electron diffraction (microED) experiments. The advent of these techniques has significantly altered the field of

structural biology, as atomic resolution structural information can now be obtained from nanocrystals (Chapman *et al.*, 2011; Shi *et al.*, 2013; Tenboer *et al.*, 2014; Kang *et al.*, 2015; Rodriguez *et al.*, 2015). However, limited access to these instruments and the preparation of crystalline samples still represents a major roadblock for experimenters.

The more common crystallographic practice of crystal microseeding may benefit from the identification and use of nanocrystals of challenging protein targets. In microseeding experiments, crushed crystals are introduced into new drops using a probe, providing nucleation sites while controlling crystal growth (McPherson, 1976; Stura & Wilson, 1991; Bergfors, 2003). This technique, along with similar adaptations such as microseed matrix screening (MMS; D'Arcy *et al.*, 2014; Ireton & Stoddard, 2004), has proven useful for the production and optimization of high-quality, diffracting crystals.

Here, we build on our previous work in identifying protein nanocrystals and employ negative-stain TEM as a tool to study and optimize crystallization in three ways. Firstly, we use a fragmentation protocol applied to crystals or nanocrystals (observed in UV-positive granular aggregates) to generate crystal fragments. The detection of Bragg spots calculated from TEM images of such fragments correlates positively with successful X-ray diffraction. Secondly, we demonstrate the

benefits of TEM to study the process of crystallization from sample analysis to estimation of solvent content to crystal optimization (*i.e.* TEM-guided crystal growth). Thirdly, we establish the usefulness of high-quality crystal fragments from crystals or nanocrystals for the growth of reproducibly larger sized crystals.

2. Materials and methods

2.1. Protein purification, crystallography and UV fluorescence screening

We utilized crystals derived from various protein samples, which were categorized into four different protein classes: (i) soluble, (ii) membrane, (iii) multi-protein complex or (iv) viral. References for protein-purification protocols and crystallization conditions are summarized in Table 1. Subsequent visual inspection of crystallization drops was achieved using an Olympus SZX16 bright-field microscope. Granular aggregates, as previously defined in Calero *et al.* (2014) and Stevenson, Makhov *et al.* (2014), and visible crystals that may be used for nanoseeding experiments were assayed for UV tryptophan fluorescence with a Jansi UVEX microscope. UV-positive samples were visualized using the *CrystalDetect* software (Jan Scientific).

2.2. Microcrystal fragmentation

Crystals were washed with a total volume of 10 μ l mother liquor to remove excess protein from the crystallization drop (this is highly recommended to prevent the formation of bubbles during vortexing). Initial fragmentation of microcrystals using a 3 mm Teflon ball (Hampton Research) and vortexing revealed minimal and highly irregular crystal fragments (see §3.1 and Supplementary Fig. S1c). To achieve better fragmentation, we utilized smaller diameter (0.1, 0.5 and 1.0 mm) glass beads (Research Products International) or

stainless-steel beads (Jinan Huawei Industry Manufacturing And Trading Co. Ltd) that are routinely used for bacterial or yeast-cell lysis. Approximately 25–30 mg of beads were placed inside a 1.5 ml microfuge tube and washed twice with water followed by stabilizing solution. Crystalline material from several crystallization drops (with over 30–50 crystals per drop ranging from 20 to 100 μ m) was added to beads on ice and diluted with 30 μ l stabilizing solution (sufficient to cover the glass beads). The beads and sample were vortexed twice for 10 s followed by centrifugation at 1000g for 30 s. After centrifugation, the solution was pipetted gently to resuspend crystal fragments and aspirated into a clean 0.5 μ l microfuge tube for subsequent experiments. 2 μ l of the crystalline solution was evaluated using bright-field and UV microscopy to determine the efficiency of fragmentation (see §3.1). TEM analysis of crystal fragments or crystal optimization *via* previously well established seeding methods (D'Arcy *et al.*, 2014; Bergfors, 2003) was performed immediately following the fragmentation protocol. Briefly, 0.3–0.4 mm loops (Hampton Research) were used to deposit ~50 nl nanoseed solution into 1 μ l crystallization drops.

2.3. Nanocrystal fragmentation

Conditions containing UV-positive granular aggregates, as previously described above and elsewhere (Stevenson, Makhov *et al.*, 2014), were selected for TEM imaging to confirm the presence of nanocrystals. To ensure sufficient material for fragmentation and TEM analysis, crystallization conditions yielding nanocrystals were repeated to generate larger volumes of nanocrystals for fragmentation experiments. Material from 4–12 1 μ l crystallization drops (depending on the granular aggregate density) containing nanocrystals was pooled and fragmented as described in §2.2 with the following caveats: (i) approximately 8–10 0.5 mm glass or stainless-steel beads were used with a total volume limited to 15 μ l stabilizing

Figure 1

Crystal fragments of Pol II-GFP. Comparison of Pol II-GFP crystal plates crushed with either (a) 0.5 or (b, c) 1.0 mm beads, illustrating the effect of bead size on nanoseed size. Using the same starting material and vortexing time (5 s), the crystals in (a) were almost completely destroyed with the use of 0.5 mm beads. Crystal morphologies such as plates or needles are highly susceptible to bead disruption and milder protocols should be attempted. Scale bars: (a) 20 nm, (b) 20 nm, (c) 50 nm. (See also Supplementary Fig. S1.)

solution and (ii) samples were vortexed for 5 s once to avoid sample destruction. Fragmented nanocrystals (referred to as

nanoseeds, since TEM analysis revealed crystal lattices with nanometre dimensions) were evaluated using bright-field and

Figure 2

TEM images of fragmented crystals. (a, d) Pol II. Fracture planes (arrows) and individual Pol II particles can be observed. (b, e) DDB1-DCAF1-UNG2-Vpr crystal fragments. (c, f) *Sindbis virus* crystals showing partially ordered (c) and disordered lattices along two different directions. (g) Crystallization of the DDB1-DCAF1 complex showed multi-nucleation that was resistant to conventional optimization protocols. (h, i) TEM images showing protein aggregation and nanocrystal nuclei growing next to crystal fragments with well ordered lattices. The use of detergents to decrease protein aggregation resulted in single crystals with improved diffraction to 3.5 Å resolution (see Supplementary Figs. S3c and S3d). Scale bars: (a) 50 nm, (b) 200 nm, (c) 50 nm, (d) 50 nm, (e) 200 nm, (f) 50 nm, (g) 50 µm, (h) 50 nm, (i) 20 nm. (See also Supplementary Figs. S2–S5.)

UV fluorescence microscopy, which was of paramount importance to qualitatively estimate the nanoseed concentration for future TEM or seeding experiments.

2.4. Fragmented crystal quantification using UV microscopy

Crystal fragments were diluted with stabilizing solution in different ratios, including 1:1, 1:2, 1:5, 1:25 and 1:125, in order to determine the seed concentration. A 5 μ l aliquot from each dilution was placed on a haemocytometer (Hausser Scientific) and covered with a 22 mm glass cover slip (Hampton Research). Bright-field and UV-microscopy images were acquired for 0.1 and 4 s, respectively, using a Jansi UVEX microscope. Crystal images collected under a nominal 15 $\times$ magnification were recorded using the *CrystalDetect* software (Jan Scientific). Manual quantification of crystal fragments was performed using the *ImageJ* (Schneider *et al.*, 2012) software plugin in *Cell Counter* (Kurt De Vos; <http://rsb.info.nih.gov/ij/plugins/cell-counter.html>) and the protocol for counting crystal fragments followed standard cell-counting procedures.

2.5. Transmission electron microscopy (TEM) experiments

All crystals generated from the proteins listed in Table 1 were subjected to analysis by negative-stain TEM. Approximately 5 μ l of nanoseeds was applied to 400 square mesh copper grids covered with a thin continuous carbon film (Electron Microscopy Sciences) and made hydrophilic by glow discharge (EmiTech) for 1 min at 25 mV under atmospheric conditions. Samples were incubated on the grid for 30 s before blotting and staining with a 2% uranyl acetate solution. Grids were mounted on a standard room-temperature specimen holder and inserted into an FEI Tecnai T12 microscope (FEI,

Hillsboro, Oregon, USA) operating at 120 keV. Images were collected on a Gatan Ultrascan 1000 CCD camera (Gatan, Pleasanton, California, USA) and FFTs were generated using

Figure 3 FFT calculation of crystal lattices shows anisotropic Bragg spots (left panels, insets) with corresponding anisotropic X-ray diffraction (right panels). (a) Pol II-GFP, (b) TFIIF and (c) Pol II-SPT4/5 complex. Scale bars: (a) 100 nm, (b) 200 nm, (c) 50 nm. Diffraction rings (resolution in Å): (d) 20, 9, 6 and 4.25, (e) 19, 8, 5.5 and 4, (f) 19, 9, 5.5 and 3.8.

the *Digital Micrograph* v.3.9.4 software (Gatan Software Team, Pleasanton, California, USA). Preparation of sample grids, data collection and evaluation of crystal images can be achieved in ~ 2 h.

3. Results

3.1. Crystal-fragmentation analysis using UV microscopy and TEM

Generally, the crystal sizes resulting from crystallization screens are too large (Supplementary Fig. S1*a*) for the direct observation of lattices by TEM, which is limited to < 3 μm along the dimension perpendicular to the electron beam (Shi *et al.*, 2013). Thus, a fragmentation protocol was established to generate crystal sizes suitable for TEM analysis (see §2.2 and

Supplementary Fig. S1). Initially, we tested the effects of various glass or stainless-steel bead diameters on crystal sizes after fragmentation. The use of 0.5 and 1.0 mm beads resulted in homogeneous populations of crystal fragments of low-micrometre sizes (Supplementary Fig. S1*b*). In contrast, the standard 3.0 mm bead yielded inhomogeneous fragmentation with large crystal sizes still present in the solution (Supplementary Fig. S1*c*), while no UV-detectable crystals were observed with 0.1 mm beads. While no differences in crystal fragmentation were observed for glass or stainless-steel beads, employing stainless-steel beads may allow a more efficient recovery of nanoseed slurry, as beads can be readily removed using a magnet.

After the presence of crystals had initially been confirmed with optical or UV microscopy, to ensure a sufficient concentration for adequate visualization, we utilized negative-

Figure 4

Crystal fragments of Pol II-GFP show isotropic and anisotropic Bragg spots along two different orientations (*a–c* and *d–f*, respectively). Lattice defects can be observed in (*d*) (arrows). (*b*, *e*) Crystal packing in space group C222₁ (unit-cell parameters $a = 221$, $b = 394$, $c = 211$ Å) illustrating the solvent-channel dimensions (indicated in Å). The preliminary model was generated from low-resolution data using molecular replacement (Rodríguez *et al.*, 2015) and rigid-body refinement in *BUSTER* (Kang *et al.*, 2015). Defects in crystal packing (*d*, arrows) and the large solvent channels (*e*) could explain the poor X-ray diffraction along the anisotropic direction in (*f*). Scale bars: (*a*) 100 nm, (*d*) 50 nm. Diffraction rings in (*c*) and (*f*) are at 15, 7.6, 5.1 and 3.7 Å resolution.

stain TEM to analyse our fragmentation protocol with 0.5 and 1 mm beads. Crystal fragments typically ranged from 50 nm to a few micrometres in length and were composed of single to multiple layers (Fig. 1). Interestingly, the use of a mild disruption protocol with 0.5 mm beads resulted in the almost complete obliteration of thin crystal plates of Pol-GFP (Fig. 1*a*). Adjusting the protocol to use slightly larger beads was necessary to obtain useful crystal fragments for TEM analysis and crystallization experiments (Figs. 1*b* and 1*c*). This suggests that bead size, quantity and vortexing time are all contributing factors that affect crystal fragment size, as fewer beads with larger diameters and shorter vortexing times yielded larger crystal fragments and *vice versa*. However, optimization of these parameters may be necessary depending on the crystal type, morphology and size.

3.2. Evaluation of crystal quality using TEM

Since crystalline lattices were clearly visualized with TEM, calculating Fourier transforms from the images allowed the qualitative evaluation of crystal lattices (Bragg spots; Fig. 2 and Table 1). In addition, it was possible to establish a correlation between the order of Bragg spots detected and the quality of subsequent X-ray diffraction data. In general, lattices with third-order or higher order Bragg spots showed diffraction at the synchrotron, whereas crystal fragments with fewer orders of Bragg spots showed poor or no diffraction (Supplementary Fig. S2).

For example, TEM analysis of crystals of the multi-protein complex comprising human damage-specific DNA-binding protein 1 (DDB1), DDB1-cullin 4 associated factor 1

(DCAF1), uracil DNA glycosylase (UNG2) and HIV Vpr (DDB1–DCAF1–UNG2–Vpr) revealed high-quality lattices (Figs. 2*b* and 2*e*), whereas initial screening of such crystals showed poor diffraction at the synchrotron (Supplementary Fig. S3*a*). The presence of well ordered lattices led us to believe that these crystals could potentially diffract to a higher resolution, which was indeed the case after optimizing the cryoprotectant conditions (Supplementary Fig. S3*b*). In contrast, poor X-ray diffraction of partially ordered lattices with low-quality Fourier transforms were observed for both *Sindbis virus* (Figs. 2*c* and 2*f*) and the parathyroid hormone receptor (Supplementary Fig. S4), a cell-surface G protein-coupled receptor (GPCR) that has proven to be challenging to crystallize. Taken together with all other protein samples tested (Table 1), these results suggest that crystallization conditions that yield higher order Bragg spots should be pursued to obtain high-resolution structural information.

In addition to lattice evaluation, TEM provided clues on sample quality and the crystallization process itself. In some instances, crystal lattices were surrounded by multiple nanocrystal nuclei (of a few nanometres in size) and protein aggregates (Figs. 2*g*, 2*h* and 2*i* and Supplementary Figs. S5*a*, S5*b* and S5*c*). In cases where high-quality lattices were observed, we tested whether the addition of additives to decrease protein aggregates may improve crystal diffraction. Our results for crystals of the DCAF–DDB1–Vpr complex indicate that such improvements are achievable (Supplementary Figs. S3*c* and S3*d*). Moreover, TEM revealed several cases of protein aggregation, filaments and nanocrystal nuclei from UV-positive granular aggregates across different crystallization conditions and samples (Supplementary Figs. S5*d*, S5*e*

Figure 5
Crystal fragments of a transcribing Pol II–Spt4/5 complex. (*a*) X-ray rastering using a highly attenuated beam showing diffracting (red) and non-diffracting (blue) areas within the crystal. (*b*) TEM images showing well ordered areas interspersed with poorly ordered areas as shown by calculation of Bragg spots. Scale bars: (*a*) 20 μ m, (*b*) 50 nm.

and S5f). The capability of TEM to differentiate crystalline material from these other particles is ideal for eliminating false positives that potentially stifle crystal optimization.

Next, we tested whether anisotropic diffraction could be detected using TEM for crystals where X-ray diffraction data showed prominent anisotropy. Anisotropic X-ray diffraction occurs when the resolution of the diffraction spots varies along the crystal axes, and severe anisotropic diffraction can have significant impact on a data set by limiting its overall resolution. Our results from three different Pol II protein complexes showed anisotropic Bragg spots by TEM, as well as anisotropic X-ray diffraction (Fig. 3). Moreover, for one such complex, for which different orientations of the crystal lattice were observed by TEM, we could visually align the crystal packing revealed by TEM with the crystal packing after solving the structure using molecular replacement (Figs. 4a, 4b, 4d and 4e). TEM images showed imperfections of the lattice along the direction of anisotropic diffraction owing to the presence of scarce crystal contacts (shown with arrows in Figs. 4d and 4f).

Another example of successfully using TEM to detect crystal lattice defects allowed us to explain uneven diffraction during crystal rastering at the synchrotron for Pol II-Spt4/5

crystals (Fig. 5a). For such crystals, a protocol for X-ray rastering was determined after discovering areas of inconsistent diffraction along the crystals as described previously (Hilgart *et al.*, 2011). TEM images of crystal fragments revealed the presence of well ordered regions interspaced with patches of blurred lattice elements (Fig. 5b). These lattice features were not typically observed for other samples.

Furthermore, lattice inspection using TEM is useful for the qualitative estimation of solvent content, in particular for crystals of large molecular-weight proteins (Fig. 6). Such samples, where relatively large solvent channels are observed, may benefit from the use of dehydration protocols to manipulate crystal solvent content and potentially improve diffraction (Tenboer *et al.*, 2014). Indeed, the use of post-crystallization dehydration protocols was paramount in the structure determination of RNA polymerase II crystals (Cramer *et al.*, 2001). Thus, we tested whether improvements in lattice quality could be visualized after the dehydration of RNA polymerase II (Pol II $\Delta 4/7$) and *Escherichia coli* dGTPase crystals. TEM analysis of pre-dehydrated and post-dehydrated crystals revealed improved lattice quality, as indicative by higher ordered, isotropic Bragg spots for both

samples (Fig. 7). In addition to improved lattice quality, higher resolution diffraction was also observed at the synchrotron for the dGTPase sample (Supplementary Fig. S6).

Lastly, since nanocrystals can commonly be found in crystallization drops (Calero *et al.*, 2014), we used TEM to determine whether it were possible to find nanocrystals with higher ordered lattices among different conditions in a crystallization screen. To this end, APOBEC nanocrystals were first identified using UV microscopy as described previously and were applied onto EM grids for visualization. TEM analysis revealed differences in crystal quality from several crystallization conditions where crystalline lattices were observed (Fig. 8). Similar to the identification of protein aggregates or filaments from UV-positive crystal drops (Supplementary Fig. S5), these data suggest the utility of TEM for providing the highest quality starting material for subsequent crystal-optimization experiments.

3.3. Quantification and nanoseeding experiments of crystal fragments

The presence of well ordered crystal lattices for various Pol II complexes (as observed by TEM) led us to utilize these crystal fragments for microseeding

Figure 6
Qualitative evaluation of the solvent content of crystal fragments. (a) Wild-type Pol II, (b) Pol II-Spt4/5, (c) dGTPase and (d) the H200Q variant of homoprotecatechuate 2,3-dioxygenase. The calculated solvent contents after Matthews coefficient calculation are 78, 65, 58 and 50%, respectively. Scale bars: 50 nm.

experiments. Traditional microseeding relies on serial dilutions of seeds used in optimization experiments to control the quality, concentration and size of the crystals that develop in the drop (Bergfors, 2003). However, these dilution series traditionally vary in seed concentration with every preparation, thus limiting their reproducibility. Given that the size of the crystal fragments was fairly homogeneous, we developed a protocol to quantify the size and number of crystal fragments using UV microscopy and a haemocytometer (see §2.4 and Supplementary Fig. S7). Using the nominal $15\times$ magnification, we were able to quantify crystals with a smallest dimension of $>2\ \mu\text{m}$ (Supplementary Figs. S7*a*, S7*b* and S7*c*). Not surprisingly, dilution series of detectable crystal fragments by UV microscopy resulted in characteristic changes in crystal size and number (Supplementary Fig. S8*a*). Moreover, implementation of a quantification protocol allowed reproducible, high-quality crystal growth for the several Pol II complexes tested, as illustrated by the overall improvement in crystal size,

resolution and mosaicity of the Pol II–TFIIB–DNA complex (Fig. 9*a*).

Although crystals with nanometre-sized dimensions were present in our fragmented samples (as visualized by TEM), it was not possible to count these crystals owing to the resolution limits of the UV microscope. Thus, we tested whether nanometre-sized nuclei contributed to the observed crystal growth during seeding experiments. The use of 0.1 mm beads for crystal fragmentation of the Pol II–TFIIB–DNA complex resulted in no detectable crystal fragments by UV microscopy, and seeding experiments using this sample did not yield detectable crystals when compared with drops seeded with the 0.5 mm bead solution over a one-week period (Supplementary Figs. S7*d*–S7*i*).

We next tested whether nanocrystals with high-quality crystal lattices as visualized by TEM could be used for nanoseeding experiments. Given the small size of nanocrystals from granular aggregates, it was not possible to count these

samples using UV microscopy; therefore, no serial dilutions were made since the concentration of nanoseeds (fragmented nanocrystals) was typically low. For the Pol II–CD3 Δ complex, $\sim 50\ \text{nl}$ of nanoseed solution was deposited into fresh $1\ \mu\text{l}$ crystallization drops as described previously. After three successive rounds of nanoseeding from fragmented nanocrystals, microcrystals identifiable by UV microscopy were produced, which were then used to generate diffracting crystals (Fig. 9*b*). Overall, these data suggest that (i) TEM-identifiable nanocrystals from granular aggregates might lead to an increasing number of conditions that promote crystal growth, (ii) UV-detectable crystal fragments provide a better medium to generate larger crystals and (iii) the quantification of such fragments leads to highly reproducible results.

3.4. Generation of crystal catalogs for XFEL experiments

To test whether we could generate homogeneously sized crystal catalogs for use at the coherent X-ray imaging (CXI) or X-ray pump–probe (XPP) stations, we utilized quantified seed solutions with varying precipitant-to-sample dilutions for the Pol II–TFIIB–DNA sample. Our results indicate that by varying the precipitant-to-protein concentration ratio and the number of UV-quantified seeds, we could control the size and quantity of the crystals (Supplementary Fig. S8*a*). Moreover,

Figure 7

Evidence of an improvement of lattice quality (order of Bragg spots) upon the implementation of crystal-dehydration protocols. (a) Pol II $\Delta 4/7$ nanocrystals before dehydration experiments, showing anisotropy. (b) Crystals undergoing dehydration (protocol adapted from Cramer *et al.*, 2001) show isotropic Bragg spots. (c, d) dGTPase crystal fragments pre-dehydration and post-dehydration, respectively, showing overall improvement of Bragg spots after dehydration. A decrease in solvent content from 63 to 58% was calculated for the different dGTPase crystal forms. The corresponding X-ray diffraction of crystals (before and after dehydration) is illustrated in Supplementary Fig. S6. Scale bars: 50 nm.

crystal drops with different ratios of protein to precipitant volume, seeded with equal amounts, generated collections of homogeneous crystal sizes (Supplementary Fig. S8*b*), while such patterns were absent for nonseeded drops. This suggests that the generation of crystal catalogs consisting of finely tuned crystal sizes may be attainable and reproducible; however, additional trials will be needed to establish a protocol.

4. Discussion

The process of crystallization remains the most significant challenge in the structure determination of macromolecules by X-ray crystallography. In a collaborative and comprehensive approach, we demonstrated the utility of TEM for the visualization, evaluation and optimization of the crystallization process. Negative-stain TEM analysis of nanocrystals or crystal fragments provides a visual method to qualitatively evaluate the characteristics of the crystal lattice and can be performed in most research institutions. In this work, we provide protocols to perform these experiments in a quick and efficient way.

Firstly, TEM images revealed details of the crystal lattice that appeared to be good indicators of the potential diffraction of the crystal at the synchrotron. Given the large number of crystals (from a wide range of 'biologically relevant' protein samples) employed in our study, it is feasible to conclude that negative-stain TEM images of fragmented crystals bearing well ordered lattices (by visual inspection) and at least third-order Bragg spots have a significant possibility of diffracting at the synchrotron (Table 1 and Supplementary Fig. S2). We provide up to 12 examples where the observation of third-order Bragg spots on nanocrystals or crystal fragments yielded at least 4–5 Å resolution diffraction. We did not observe useful X-ray diffraction for crystals that had disordered lattices of less than first-order to second-order Bragg spots. Moreover,

this correspondence was also observed for crystals that diffracted anisotropically (Fig. 3). Thus, the qualitative information gained by analysis of nanocrystals or crystal fragments should allow the experimenter to decide whether a crystallization condition should be explored further and optimized or discarded, saving valuable time during crystallization efforts.

A physical reason for such behavior might be that highly ordered lattices could better withstand the effects of negative staining (*i.e.* dehydration, the damaging effect of the stain itself and imaging at room temperature). Such behavior may explain the anisotropic diffraction observed for Pol II-GFP crystals in Fig. 4. It is possible that the larger number of crystal contacts along the isotropic direction could withstand the effects of crystal dehydration while the direction with fewer contacts collapses. Conversely, we believe that the fragmentation protocol itself probably has little effect on the quality of the lattice for two reasons: (i) protocols for fragmentation of crystals for nanocrystallography at free-electron lasers have been employed and such crystals diffract to a comparable resolution to their larger counterparts (Redecke *et al.*, 2013; Stevenson, DePonte *et al.*, 2014) and (ii) lattice comparison of grown nanocrystals with fragmented microcrystals show no difference (in terms of quality) when visualized with negative-stain TEM (Abdallah *et al.*, 2015).

Secondly, we demonstrated the utility of TEM to identify crystal pathologies that contribute to poor growth and X-ray diffraction data. Similar to experiments utilizing atomic force microscopy (McPherson *et al.*, 2000; Kupitz *et al.*, 2014), crystal pathologies revealed by TEM included: (i) crystal 'contamination' by heavy protein aggregates and micro-crystal nuclei (Figs. 2*h* and 2*i* and Supplementary Figs. S5*a*, S5*b* and S5*c*), (ii) anisotropic diffraction (Fig. 3) and (iii) crystal lattice defects (Figs. 4*d* and 5*b*). Detection of lattice defects in some crystals could point to the presence of samples containing protein contaminants, aggregates or partially proteolyzed protein as well as discrepancies in the stoichiometry of the sample.

Similarly, identifying crystals that possess anisotropic diffraction may indicate that steps to improve crystal contacts, such as altering or adding reagents to the crystallization conditions (Sleutel & Van Driessche, 2014) or modification of the protein itself, may be advisable. The presence of heavy protein aggregation or nanocrystal nuclei indicates that increased protein solubility or decreased crystal nucleation may be useful to improve diffraction.

Thirdly, TEM enabled a qualitative estimation of crystal solvent content and permitted us to explore the effect of lattice dehydration on crystal diffraction. This application was particularly noteworthy since (i) crystal-dehydration protocols have proven to be very useful in the improvement of X-ray diffraction

Figure 8
Crystal fragments of APOBEC obtained from two different crystallization conditions. (*a*) 1.5 M sodium citrate, (*b*) 200 mM magnesium acetate + 10% PEG 4000. Scale bars: 50 nm. The crystals in (*a*) show a poorly ordered lattice with no Bragg spots, while the crystal lattice in (*b*) shows second/third-order Bragg spots, which may be indicative of a condition which will produce high-resolution crystals.

(Tenboer *et al.*, 2014; Heras & Martin, 2005) and (ii) negative staining with uranyl acetate provides a very high contrast between solvent channels and biological macromolecules. Furthermore, decreasing the solvent content for Pol II $\Delta 4/7$ and dGTPase crystals (decreases of 13 and 5% as calculated from the Matthews coefficient, respectively) showed an improvement in the order of Bragg reflections (Fig. 6) which correlated with improved X-ray diffraction (Supplementary Fig. S6). Although our assessment of solvent content was qualitative, TEM images provide a means to accurately estimate crystal solvent content through evaluation of the crystal symmetry observed in FFTs and measured protein particle size, as described elsewhere (Brown *et al.*, 1988). Such information could potentially guide post-crystallization protocols to help improve X-ray diffraction.

Furthermore, our work demonstrates a comprehensive approach to seeding through (i) TEM identification and evaluation of crystal lattices and (ii) the use of UV microscopy to quantify seed concentration using conventional cell-counting protocols. While the use of granular aggregates has

provided suitable starting materials for microseeding experiments (D'Arcy *et al.*, 2014), the success of applications such as MMS will benefit greatly from methods which are capable of detecting crystalline material. Semi-automated applications such as SONICC and UV fluorescence techniques are capable of visualizing nanometre-sized crystals, but they lack the ability to provide information on the quality of the starting seeding material. For instance, crystallization experiments using Pol II-TFIIF, PTHR1 and *Sindbis virus* yielded nanocrystals and microcrystals for all samples; however, TEM analysis allowed the identification of lattice defects (Figs. 2c, 2f, 3b and Supplementary Fig. S4), which suggested that these crystals were unlikely to succeed as seeding nuclei.

In contrast, nanocrystals identified by TEM from a crystallization screen for the Pol II-CD3 Δ complex which showed second-order Bragg reflections (Stevenson, Makhov *et al.*, 2014) produced diffraction-quality crystals after multiple rounds of iterative microseeding (Fig. 9). Cases such as these suggest that exploring multiple conditions within a screen may lead to higher quality starting material for microseeding

Figure 9
Bright-field images of microseeding experiments with corresponding post-seeded diffraction. (a) Microcrystals (left panel) of a complex composed of Pol II, TFIIB and a nucleic acid scaffold were fragmented and used for microseeding experiments to obtain larger, well ordered crystals (middle panel) which showed high-resolution diffraction (right panel). (b) Nanocrystals from granular aggregates (left panel) of a complex between Pol II and CD3 Δ were used to generate nanoseeds used in nanoseeding experiments to generate microcrystals (middle panel) which diffracted to high resolution at the synchrotron (right panel). Scale bars: (a) 100 μm , (b) 50 μm . Diffraction rings: 15, 7.6, 5.1 and 3.7 $\text{\AA}$ resolution. (See also Supplementary Figs. S7 and S8.)

experiments to optimize crystal growth. Ideally, an analytical procedure involving identification by SONICC or UV fluorescence of protein nanocrystals followed by scoring through TEM analysis would greatly aid the crystallographer. Indeed, such a procedure was utilized after UV-fluorescence identification of APOBEC crystals from a matrix crystallization screen. TEM analysis of crystals generated from different crystallization conditions demonstrated the utility of TEM in distinguishing ordered crystal lattices from crystals with lattice defects (Fig. 8). In our experiments, a direct correlation between seed quality and the appearance of high-quality crystals in seeded drops was easily established and corroborated by X-ray diffraction data (Fig. 9).

Finally, critical to our TEM analysis was the development of a crystal-fragmentation protocol that produced homogeneously sized crystal fragments. While fragmentation protocols have been used to generate homogeneously sized crystals that diffract to high resolutions at XFEL sources (Redecke *et al.*, 2013), homogeneous sample preparation for both XFEL and microED techniques is an active area of research (Abdallah *et al.*, 2015; Conrad *et al.*, 2015; Fromme *et al.*, 2015; Ibrahim *et al.*, 2015). Quantification of crystal fragments by UV microscopy allowed highly reproducible seeding experiments (Fig. 9a) and the generation of homogeneously sized crystal catalogs through manipulation of crystallization conditions (Supplementary Fig. S8). Application of this protocol to batch crystallization methods, such as those commonly used for sample preparation in SFX or microED experiments, would likely generate the high-quantity uniform crystals required for these experiments. Moreover, such experiments can be complemented by TEM analysis to ensure sample homogeneity, monodispersity and quality prior to conducting experiments, thus increasing the chances of success.

5. Conclusion

In this work, we established a reproducible protocol using TEM to qualitatively visualize fragmented crystals with nanometre-to-micrometre dimensions to study details of the crystallization process. TEM analysis allows the direct comparison of the quality of crystal lattices among different crystallization conditions, thus guiding the experimenter's selection of crystallization conditions that promote well ordered crystal growth and high-resolution diffraction. In order to probe the real limits of diffraction of such nanocrystals or crystal fragments, TEM experiments using cryo methods and the use of special detectors to probe electron diffraction would be needed. Overall, we believe that the screening methods described here provide a rationalized approach to meet the challenges associated with obtaining suitable crystals for macromolecular-crystallography experiments at both synchrotron and XFEL sources.

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