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UV microscopy at 280 nm is effective in screening for the growth of protein microcrystals

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This paper describes the relatively simple modification of a light microscope to operate with ultraviolet-based optics. Using a 280 nm UV illumination source, protein microcrystals are readily visualized in gels made from hydrated bilayers of phospholipids. Non-colored proteins stand out as clearly as colored proteins in this system, the imaging of which is based on UV absorption by tryptophan residues. In addition, protein crystals are easily distinguished from salt crystals. Artifacts from the lipid-based crystallization medium, which are frequently seen in brightfield microscopy, are greatly reduced when viewed in this UV-based microscope.

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1. Introduction

Detection of protein microcrystals that may form during the early stages of crystallization trials remains a challenging part of crystallographic research. Crystals can be obscured by optical debris that forms within the setup, and there always exists the question of whether a crystal is protein or salt. In membrane protein crystallography, crystals are often quite small, and screening for growth of membrane protein crystals thus presents an even greater challenge. It is not uncommon that crystals need to be seen reliably at sizes ranging down to 5 µm or less.

When crystals of this size are grown in hydrated lipid bilayer gels (Caffrey, 2003; Rouhani *et al.*, 2002), optical disturbances from non-uniformities in the lipid phase can often obscure crystal detection. Protein crystals formed from colored proteins, such as bacteriorhodopsin, may still be relatively easy to detect within the lipid-gel background. Microcrystals of non-colored proteins can also be seen if the setup is optically isotropic, but in practice they have a greater tendency to be obscured by 'texture' in the lipid matrix. Accurate discrimination between true protein microcrystals and crystals of salt or other components in the setup is a key aspect of efficient screening. When using lipid-based crystallization media, there is the added possibility that lipid crystals may form that are birefringent and appear quite protein-like, as we have noted in our work with monoolein (Rouhani *et al.*, 2002). In hydrated lipid gels of phosphatidylcholine, growth of birefringent crystals or aggregates which are thought to be lipid has also been frequently observed (Faham & Bowie, 2002).

Microscopes that are designed or modified for use with ultraviolet optics have been well described in a prior review (Heimann & Urstadt, 1990). The semiconductor industry, in particular, has extensively used deep UV microscopes for defect analysis. In addition to enhanced resolution, UV light provides a significant improvement in image contrast for materials that are transparent or translucent under visible light but opaque to UV. Protein crystals are expected to be opaque to UV because they absorb 280 nm light due to their

tryptophan residues. This feature should generate sufficient contrast to easily see protein *versus* non-protein components. The high density of protein within a crystal, in the region of 500 mg ml⁻¹, is further expected to provide a clear distinction between crystals or precipitates and the soluble protein that remains in the crystallization medium.

This paper describes the modification of a light microscope to operate with ultraviolet-based optics at 280 nm for the visualization of protein crystals. It is demonstrated that non-colored protein crystals can be seen as easily as one can see crystals that are colored, including crystals formed in hydrated bilayers of phospholipids. Furthermore, protein crystals are shown to be easily distinguished from salt crystals.

2. Apparatus

A Zeiss UEM microscope was modified to accommodate 280 nm UV optics (Fig. 1). This was accomplished by removing all optical elements from the microscope tube, including the eyepieces, mirrors and filters. In effect, the tube was converted into a single-lens microscope consisting of the UV objective and a UV-sensitive camera. This approach is possible as long as one uses 160 mm objectives (DIN standard), as they do not require a lens downstream from the objective as do infinity-corrected optics. The entire condenser assembly was also removed in order to thread a UV light-guide through a hole underneath the microscope to a position directly below the stage.

The illumination source was a Hamamatsu LC4 (Bridgewater, NJ, USA) with 365 nm reflector powered by a 150 W ozone-free xenon lamp connected to a 5 mm diameter quartz light-guide. A 280 nm narrow bandpass filter was rigged approximately 5 mm from the end of the light-guide. This filter has a bandpass of 10 nm and permits 18% transmittance of the specified wavelength, while sharply excluding all others (280FS10-25, Andover Corp., Salem, NH, USA). This light-guide plus filter assembly is mounted by an adjustable

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clamp directly underneath the sample. The adjustable nature of the assembly permits exchange between the UV source and a visible light source. The visible source is a white LED flashlight on a flexible neck (Coast #LL7583, Portland, OR, USA), which has a well focused beam and can be easily directed onto the sample.

The choice of objective is critical for deep UV transmission. The primary type of objectives that are compatible with 280 nm light are those made from a combination of quartz (fused silica) and calcium fluoride. Zeiss manufactured a line of objectives called Ultrafluor, which work very well for this application. In the initial work reported

here, an Ultrafluor 32/0.40 Glyc 160/0.35 Qu was used. As mentioned above, the objective is the only element required between the sample and the camera.

The camera used in this study was a Quantix 1401E with Metachrome II coating from Photometrics (Tucson, AZ, USA). The Metachrome II coating maintains quantum efficiencies (QE) of 10% from 450 nm down to 200 nm, the spectral region in which the QE for a normal CCD will fall off rapidly. The flat QE curve in the UV region is important for proper imaging, as a camera which is more sensitive to 300–450 nm light than 280 nm light loses the contrast of 280 nm protein absorption due to brighter illumination from incident fluorescence emission.

The UV microscope behaves similar to a standard microscope illuminated with visible light. Focusing is routine with a suitable and constant depth of field, and multiple samples can be viewed without significant modification to the focus.

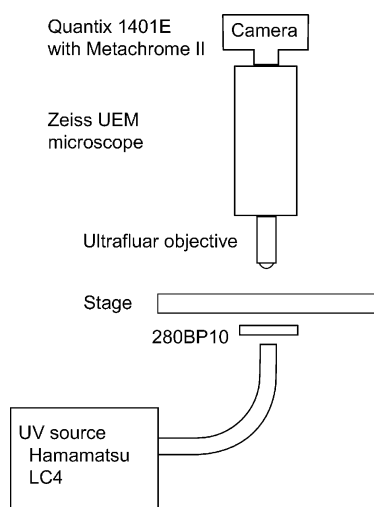


Figure 1
Schematic of a microscope modified for 280 nm ultraviolet protein crystal visualization. Details are described in the text.

3. Results

Protein crystals of bacteriorhodopsin (bR) were grown in phosphatidylethanolamine-based connected-bilayer gels. [The development of this and other PE lipid–gel media and a description of methods used for screening conditions that produce the growth of protein crystals will be detailed elsewhere (Rouhani *et al.*, in preparation).] Detergent-solubilized wild-type bR was reconstituted into gels of monomethyl-DOPE with 5% DOPE-mPEG350. Aliquots of this gel were dispensed, along with 2.5 M sodium potassium phosphate pH 5.6, into a sandwich of thin plastic sheets made of polychlorotrifluoroethylene (PCTFE or Aclar, Honeywell Specialty Films, Morris Township, NJ, USA). PCTFE has excellent chemical resistance and

vapor barrier properties, and the Aclar33C type has good optical properties, including minimal absorbance of 280 nm light. The sandwich is constructed with a 190 μm bottom layer of Aclar33C, then 100 μm of ultraclean acrylic adhesive (502 FL, 3M, St. Paul, MN, USA) with 6 mm diameter cutouts for the sample, and a 50 μm top layer of Aclar33C. The plastic assembly is also beneficial as samples containing protein microcrystals can be cut out and mounted directly on the beamline. Diffraction of protein crystals is not significantly impacted by Aclar33C at these thicknesses.

Fig. 2 compares images of bR crystals recorded (i) with a stereomicroscope (Olympus SZX-12) and (ii) with an Ultrafluor 32/0.40 Glyc 160/0.35 Qu on the modified Zeiss UEM microscope. In bright-field (Fig. 2a), bR crystals appear as purple hexagons in a range of sizes, with the largest crystal in the field being 50 μm across. Figs. 2(b) and 2(c) compare images with the Ultrafluor 32 $\times$ under visible and UV illumination, respectively. UV light appears bright to the camera, while protein absorbs UV and thus appears dark.

Bacteriorhodopsin crystals appear considerably more distinct in the modified microscope when viewed under UV imaging conditions than they do under visible-

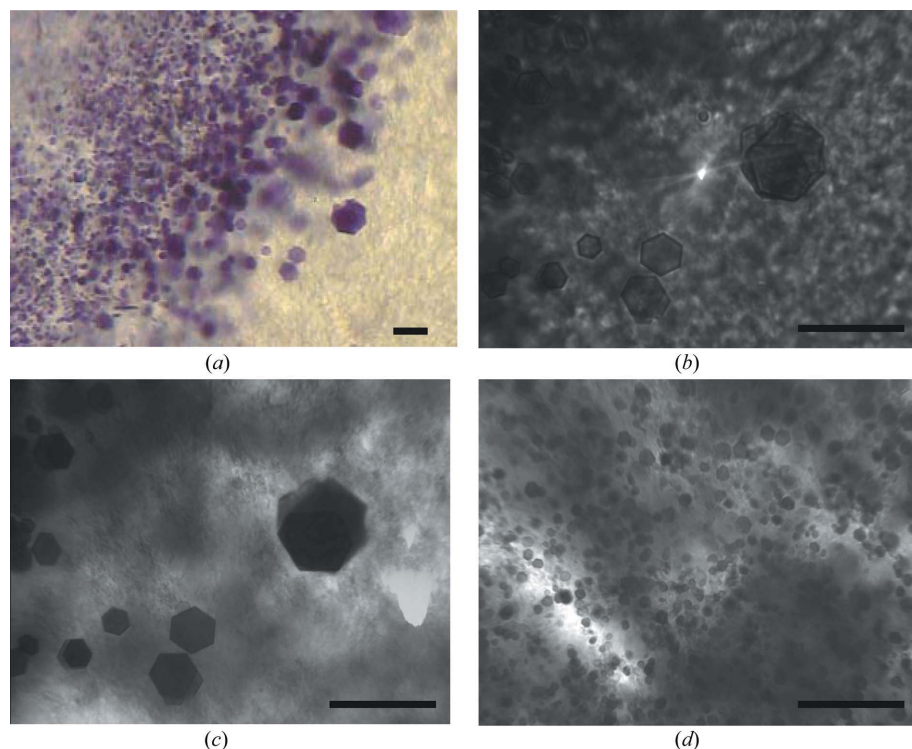


Figure 2
Protein crystals of bacteriorhodopsin grown in a PE-based bilayer gel, imaged under both visible and UV light. (a) Brightfield on an Olympus SZX-12 stereomicroscope. (b) White LED illumination with Ultrafluor 32 $\times$ on a modified Zeiss UEM microscope. (c) 280 nm UV illumination with Ultrafluor 32 $\times$ in the same frame as (b). (d) 280 nm UV illumination with Ultrafluor 32 $\times$ of the smallest crystals in the field, which are 2–5 μm across. Scale bars represent 50 μm .

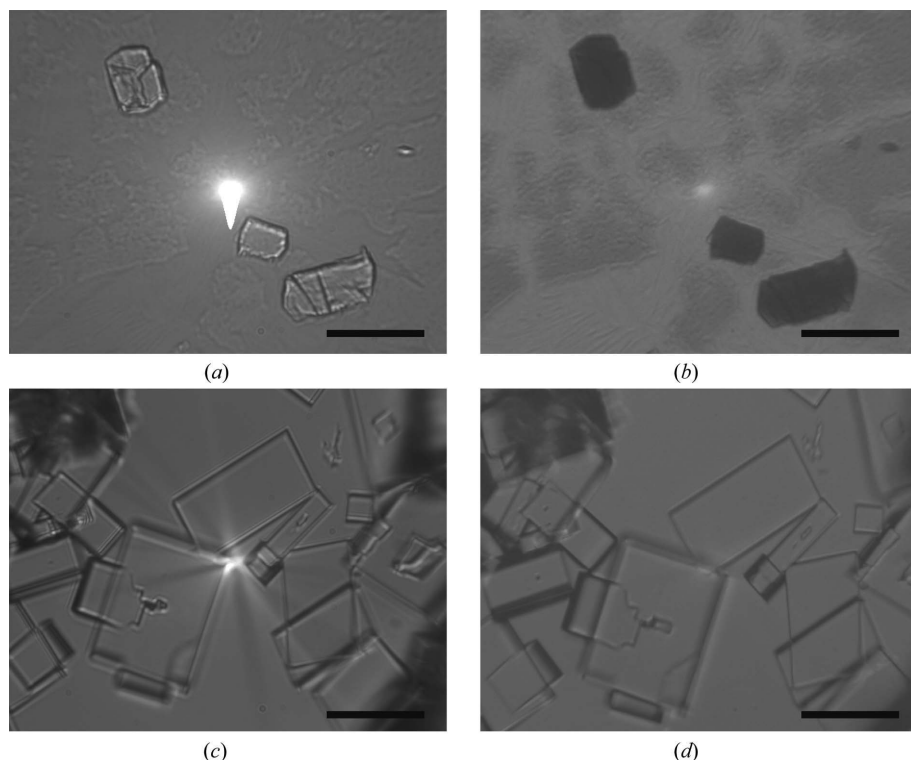


Figure 3

Lysozyme and sodium chloride crystals are compared under visible and UV light. (a),(b) Lysozyme crystals grown within a PE-based bilayer gel. (c),(d) Sodium chloride crystals formed by dehydrating a 5 M solution. The left panel, (a) and (c), is illuminated with white LED light and the right panel, (b) and (d), with 280 nm UV light. All are viewed with an Ultrafluor 32× objective. The scale bar represents 50 μm.

wavelength imaging, with non-crystalline protein clearly seen but not interfering with detection of crystals. Fig. 2(d) shows an area of the sample which has large numbers of the smallest crystals in the field. Crystals 2–5 μm across can be clearly detected against a complex background. Camera exposure times can be extended, if necessary, to see through a concentrated area of protein. The exposures in these examples range from 0.2–1 s duration. Lipid–gel ‘texture’, which is a problem even for these colored proteins under visible light, is very substantially reduced under UV light. The UV microscope thus circumvents one of the more negative attributes that is often associated with connected-bilayer crystallization media.

Next, the microscope was tested with non-colored protein crystals to verify that colored and non-colored proteins are imaged similarly. Lysozyme was mixed in the phosphatidylethanolamine gel, dispensed with crystallization solution onto a quartz slide, and lightly squashed into a thin layer by adding a coverslip made of cyclic olefin copolymer (Topas 8007-D62 COC, Ticona, Auburn Hills, MI, USA). This coverslip transmits the majority of incident light at 280 nm and has a refractive index the same as glass. Figs. 3(a) and 3(b) compare images obtained with the Ultrafluor 32× under visible and UV illumination, respectively. It is obvious that the crystals are lysozyme as they are dark due to UV absorption by tryptophan residues. Their non-standard appearance is likely due to both desiccation and being grown within the aqueous channels of the lipid gel. In contrast, sodium chloride crystals appear nearly the same under visible (Fig. 3c) and UV light (Fig. 3d), since the salt crystals do not absorb at 280 nm.

4. Discussion

The images presented above demonstrate the proof-of-principle for a technique that offers valuable improvements over current methods

for identifying protein crystals. UV imaging offers particular advantages for membrane protein crystals, which tend to be smaller and more easily obscured when growing in an optically heterogeneous environment.

When using colored proteins, such as bacteriorhodopsin, the detection of protein microcrystals is manageable, even in optically heterogeneous media, but with non-colored proteins it becomes more challenging. When screening, there is a great likelihood that one might miss some promising ‘hits’ when observing hundreds of samples. Using UV-based imaging, this becomes less of a concern because non-colored protein crystals can now be visualized at least as easily as one can see crystals that are colored under visible light. This is a significant concern with membrane protein crystals grown in bilayer gels, as initial hits may only appear as very small crystals before the conditions have been optimized.

The modification of the microscope that we have used is a relatively simple one, and it should be possible to replicate a similar arrangement on many types of older, 160 mm tube-length instruments. The key components are a UV light source, a compatible objective, and a UV-sensitive camera. Without too much cost or difficulty, any crystallography laboratory or beamline

should be able to create a similar microscope for protein-crystal identification. Since the microscope is already modified for camera acquisition only, it is a short step to consider automating image capture for screening. UV imaging should likely increase the proficiency of crystal scoring algorithms.

The images presented here were obtained with an Ultrafluor 32× objective. For routine screening, a lower power objective would be better in order to accommodate the full field-of-view of the sample. In fact, an Ultrafluor 10× objective was tested on the system, which increased the field-of-view threefold, and shortening the microscope tube-length increased it up to a further threefold, with no apparent negative effect on the optics.

Working with UV wavelengths presents unique concerns. Attention to safety is important since 280 nm light is hazardous to the skin and eyes. Also important is the choice of container within which crystals are grown, as all such materials must transmit at 280 nm. Most of the traditional screening plates use glass or polystyrene in the light path. These materials strongly absorb at 280 nm, effectively blocking light from illuminating the sample. Some of the newer crystallography plates use special plastics which are known to transmit UV light and should be amenable to this method. Alternatively, custom crystallization formats can be developed using special thin plastic films.

Consideration must also be given to possible UV damage to the protein in the crystal setups that are being screened. Repeated or lengthy observations of the samples may have a detrimental effect. In the instrument described above, the amount of 280 nm light at the sample is estimated to be 1 mW cm⁻², which is approximately 50 times the UV intensity in a spectrophotometer. These considerations will need to be explored in more detail. In the worst case, screening by UV microscopy can at least identify promising hits in sacrificial

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setups, so that attention can be productively directed to replicate setups by visible-light microscopy.

An example of a different type of UV imaging system is installed on Beamline BW7b at the EMBL outstation in Hamburg, Germany (Pohl *et al.*, 2004). In that case, the fluorescence emission from 280 nm excitation is imaged. Intrinsic fluorescence from tryptophan residues exhibits maximum emission between 330 and 370 nm. UV-based fluorescence imaging is described to work effectively for screening crystal setups, when an appropriately modified microscope is used (Judge *et al.*, 2005). An advantage of the UV absorbance method presented in this paper is that every absorbed photon produces contrast. This permits shorter exposure times, which are preferred in order to minimize possible UV damage.

In summary, protein crystals display strong contrast *versus* background when imaged with 280 nm ultraviolet light, even in complex crystallization media such as connected-bilayer lipid gels. Images of colored and non-colored protein crystals are the same under UV light. In addition, protein crystals can be easily distinguished from salt crystals. The imaging resolution permits crystals as small as 2 μm to be seen in a complex background. The microscope modification

needed for this purpose is straightforward and should be easily replicated in-house by any crystallography laboratory or beamline. The ability unambiguously to detect and identify protein micro-crystals by microscopy has significant value in protein crystallography.

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