calculation. However, the coarsest scans can be seen to miss some minor secondary peaks in some cases that can sometimes, in fact, represent a true solution or correspond to the orientation of a more minor fragment in the structure and so are important.

(iii) The minimum $\Delta\theta$ step size required in such calculations is of the order of 1–2° in the examples studied, much as expected.

(iv) In the case of 3-methylxanthine (Low, Tollin, Brand & Wilson, 1986), mentioned above as a case where the Patterson-method solution did not routinely refine to a full structural solution, the model obtained from the optimized rotation-function calculation did in fact refine routinely, evidence of a substantive advantage in using the present procedure.

Concluding remarks
The simple optimization procedure proposed for rotation-function calculations appears to yield more efficient calculations but, more importantly, it gives more cross checks on the results of these calculations, a good estimate of their true angular resolution and, in difficult cases, a quantitatively more correct orientation. All of these aspects assume more importance when one considers the increasingly elaborate use being made of rotation-function calculations.

References


A fast and portable microspectrophotometer for protein crystallography. By Andrea Hadfield* and Janos Haidu†‡ Laboratory of Molecular Biophysics and Oxford Centre for Molecular Sciences, Oxford University. The Rex Richards Building, South Parks Road, Oxford OX1 3QU, England

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Abstract
Spectroscopic measurements on crystals during X-ray data collection provide additional information on the composition of the crystal and can be used in the interpretation of structural data. This paper describes a portable microspectrophotometer to obtain UV–visible–near-IR spectra from single crystals during X-ray measurements. The instrument consists of a deuterium lamp, optical fibres, a pair of mirror lenses and a monochromator equipped with a photodiode array detector. Spectra can be recorded in short periods of time (a few milliseconds) from a measurement area of 0.10 mm diameter or smaller. The device can be used to monitor spectral changes in crystals during time-resolved X-ray experiments so that the X-ray camera can be triggered at the right moment as determined by the spectrum, thereby eliminating much of the present guesswork from such studies.

Introduction
Single-crystal absorption studies on protein crystals provide detailed electronic spectra on chromophores and on the equilibrium and kinetic constants of ligand binding and catalysis (Eaton & Hochstrasser, 1967; Rossi & Bernhard, 1970; Makinen & Eaton, 1973; Vas, Berni, Mozzarelli, Tegoni & Rossi, 1979; Mozzarelli et al., 1982; Mozzarelli, Peracchi, Rossi, Ahmed & Miles 1989; Mozzarelli, Rivetti, Rossi, Henry & Eaton, 1991; Kuo, Lipscomb & Makinen, 1986; Makinen, Hill, Zepppezauer, Little & Burdett, 1987; Makinen, Zelano & Troyer, 1987; Metzler et al., 1988; Rossi, Mozzarelli, Peracchi & Rivetti, 1992; Rivetti, Mozzarelli, Rossi, Henry & Eaton, 1993). Commercial microspectrophotometers require the crystals to be specially mounted, restricting access to the sample for measurements from other directions. The large size of these devices and their extreme sensitivity to mechanical perturbations makes in situ spectrophotometric measurements in X-ray cameras difficult. The apparatus described below is small, portable and leaves the crystal accessible for a wide range of experiments including X-ray or neutron measurements.

The instrument
Fig. 1 shows the layout of the instrument. The light source (1, 2) can be chosen to match the spectral region of interest. In most of our studies, a portable deuterium lamp was used with a built-in fibre-optics connection (30 W lamp, model DTL200 from Guided Wave, 5190 Golden Foothill Parkway, El Dorado Hills, CA 95630, USA). Single-core sheathed quartz fibres (0.5 mm diameter, 2 m long, supplied by Guided Wave, catalogue no. C2-2B) are used throughout the apparatus to reduce artifacts caused by...
variations in the spectral properties of fibres in fibre bundles. The lamp provides good spectral intensities in the UV-visible range, making it a suitable instrument for studies on proteins and other macromolecules. A filter can be inserted between the light source and the optical fibre in order to cut out the hardest part of the UV radiation (below 250 nm) and prolong the lifetime of the optical fibres. Light emerging from the tip of the first quartz fibre is collimated by two quartz lenses \((f = 25 \text{ mm}, \text{ diameter } = 0.5 \text{ in}; \text{ Oriel, 250 Long Beach Boulevard, PO Box 872, Stratford, CT 06497, USA, catalogue no. 41220})\) fixed to the back of the first mirror lens (magnification 15 \(\times\), working distance 24 mm; Ealing Electro-Optics Plc, Greyecaine Road, Watford WD2 4PW, England, catalogue no. 25-0506). The fibre can be translated along the optical axis of the assembly for alignment. Light passing through the sample is picked up by a second (and identical) mirror lens in a symmetrical arrangement. This lens is also fitted with similar quartz collimating lenses. Both mirror lenses are mounted on \(XYZ\) stages on a mounting pod (9). Mirror lenses were chosen for the apparatus because they reflect light over the full wavelength range that might be used in UV-visible-near-IR spectroscopy and focus the entire spectrum into a single spot without chromatic aberration. Chromatic aberration by the low-magnification quartz collimating lenses is negligible compared with the diameter of the fibre core (0.50 mm) and the size of the measurement spot (0.10 mm). The diameter of the measurement area can be changed between 0.10 and 0.02 mm with the aid of an iris diaphragm placed between the mirror lens and the quartz collimating lenses (not shown). Note that the secondary mirror within these reflecting objectives blots out 17.5\% of the light entering the Ealing lenses. However, the alignment of the blind spots within the assembly ensures a maximum transmission of 82.5\% for the combined system.

Light from the receiving objective is fed into a 125 mm grating monochromator (item 7, supplied by Oriel, 250 Long Beach Boulevard, PO Box 872, Stratford, CT 06497, USA, catalogue no. 77400). The monochromator can be fitted with a variety of gratings to span various spectral ranges. The grating best suited for work on protein crystals is a UV-blazed grating with 400 lines per mm. This gives a useful spectral band width of 530 nm on the detector. The grating can be rotated, allowing the experimenter to move the 530 nm wide observation frame anywhere between 200 and 800 nm. The present system limits this range to 250–800 nm. Different fibres, collimator lenses and light sources would permit measurements in other spectral ranges (e.g. IR). Spectra are recorded on a 512-element photodiode array detector (item 8, Oriel Instaspec 1B, catalogue no. M-7714B). Other types of diode arrays could also be used. The system is interfaced with a lap-top computer (Toshiba 5200), which controls the detector and can drive other devices like flash lamps, goniometers, camera shutters etc.

**Alignment and spectral calibration**

Alignment of the system is done sequentially starting from the light source in four steps: (i) maximizing light output from the deuterium source; (ii) alignment of the tip of the second fibre with the entrance slit of the monochromator; (iii) adjustment of the collimator lenses at the back of the mirror lenses by alternately connecting each mirror lens to the light source; (iv) adjustment of the illuminator and collector mirror lenses relative to the sample and to each other on the mounting pod. A single photodiode in a fibre-optics housing may be used for maximizing light throughput during the coarse alignment of the system. Fine tuning is accomplished by using the spectrophotometer itself in a 'loop mode', i.e. performing continuous measurement cycles.

Wavelength calibration of the monochromator and detector can either be done with a small mercury pen light...
Oriel, catalogue no. 6035 placed in a small fibre-optic housing, catalogue no. 6058) or with calibration filters (e.g. holmium) in connection with a white light source.

Assessment of linearity
The linearity and the useful dynamic range of the instrument was evaluated experimentally. We found that X-ray film absorbs light evenly across a wide wavelength range. A step wedge was constructed from increasing numbers of lightly fogged unexposed but developed X-ray films (CEA Reflex 25). The thickest part contained 11 layers. Figs. 2(a) and (b) show the response of the spectrophotometer in a wide spectral range. The response is linear up to about 2.2–2.40 OD. At high optical densities, the stray light effect needs to be taken into account (Mehler, 1954). Note that graphs like Fig. 2(b) can be used to calibrate measurements in regions where response is not linear.

Taking spectra from crystals
It is important that the crystal fills in the measurement area completely. Fig. 3 shows the reproducibility of spectral measurements on a crystal of glycogen phosphorylase b. In the figure, 20 spectra are superimposed. Spectra obtained with the device are reproducible to within 0.0025 OD above a background of 1.0 OD throughout the spectral range (250–800 nm), provided the crystal is kept in a fixed position. There is, however, a considerable variation in the intensity of spectra depending both on the orientation of the crystal (a prism effect) and the thickness of the material in the light beam. This is a common feature to all microspectrophotometric measurements and must be compensated for in the experiment. Protein crystals mounted in quartz capillary tubes may pose a further problem because the capillary acts as a cylindrical lens. Moving the capillary relative to the optical axis can alter the measurement values, especially when the tube is filled with liquid. These effects can be compensated for: accurate and reproducible measurements of difference spectra require that both the reference spectrum and all subsequent data spectra are recorded from the same part of the crystal at the same crystal/tube orientation. This means that, during X-ray data collection, the crystal has to be rotated back to the position of the reference measurement for subsequent spectral measurements. Note that reference spectra can be recorded for many crystal orientations and the data can be stored. Difference spectra so obtained are highly accurate and reproducible.

Concentration measurements in crystals
Measurement of concentrations in crystals needs careful calibration in order to determine the effective optical path length through the sample. Ideally, but not necessarily, the crystal should be centred in the capillary tube and the axis of the tube should intersect the optical axis. This minimizes artifacts caused by the rotation or translation of the capillary tube. A reference spectrum should be taken from just above or below the crystal. The effective optical
path lengths can be determined in a flow-cell arrangement using an inert calibration solution of known optical density and spectral properties. The chromophore has to be a substance that does not interact with the protein and cannot diffuse into the body of the crystal. We suggest using coloured macromolecules, like Blue Dextran, haemoglobin, myoglobin etc. The spectrum of this calibration solution should be recorded separately, e.g. in a conventional cuvette.

Before the introduction of the chromophore into the flow cell, two reference spectra are recorded: one from the crystal and one from the capillary filled with the usual crystal buffer without the chromophore. In the next step, this solution is replaced by a similar solution that now contains the chromophore (the calibration solution). Spectra are then recorded from identical positions on both the crystal and the tube. As the concentration of the chromophore in the calibration solution is known, these measurements permit the calculation of the 'effective optical thickness' for the crystal in the given orientation using Beer's law. Accurate concentration measurements can then be performed in subsequent experiments provided the crystal is kept in the orientation of the calibration measurements.

The system described in this paper has been designed for applications in crystallography. However, it can be used in a much wider range of experiments where small samples have to be analysed in confined spaces. Fig. 4 shows successive spectra taken on a 0.4 µl solution of 3,5-dinitrophenol phosphate in a photochemical experiment. The solution was sealed in a quartz capillary tube and was photolysed by a 1 ms long light pulse from a Xenon flash lamp (Rapp & Guth, 1988). The resulting products are 3,5-dinitrophenol and phosphate. The reaction was followed by measuring the concentration of 3,5-dinitrophenolate (pKa = 6.8) at 400 nm (ε = 2800 M⁻¹ cm⁻¹; Parke, 1961).

Concluding remarks

The apparatus makes established spectrophotometric techniques available for a wider range of experiments. The useful linear range of the instrument and the reproducibility of the measurements is better than those of currently available commercial devices. The cost of building this microspectrophotometer (excluding the lap-top PC) is about £20 000 compared with about £100 000–200 000 for a commercial microspectrophotometer.

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References