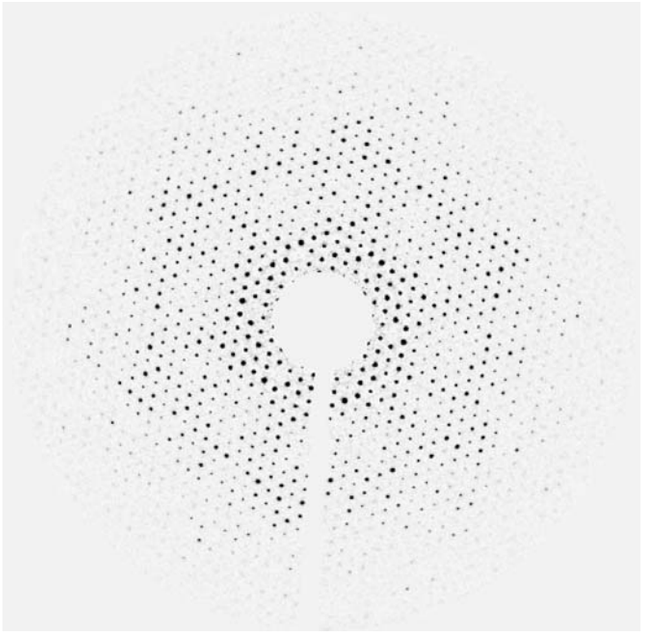

Fig. 19.2.3.1. Electron diffraction pattern of trehalose-embedded bacteriorhodopsin, with Bragg reflections extending to 2.5 Å. The unit-cell parameter of this 45 Å-thick membrane protein crystal is 62.5 × 62.5 Å arranged in a *p*3 two-dimensional space group. The raw diffraction pattern was recorded on a Gatan 2k × 2k CCD camera with 300 kV electrons in a JEOL 3000 electron cryomicroscope equipped with a field emission gun and a liquid-helium (4 K) cryoholder. The pattern displayed has been contrast-enhanced using radial background subtraction. A central beam stop was used to prevent saturation of the detector but has blocked off some reflections. $R_{sym}$ for the Friedel-symmetry-related reflections (about 290 pairs) was computed to be about 5%. (Courtesy of Drs Yifan Cheng and Yoshinori Fujiyoshi at Kyoto University.)

### 19.2.3.2. *Radiation damage*

All protein crystals are prone to radiation damage caused by inelastically scattered electrons (Glaeser, 1971). This physical process is easily seen in the fading of electron diffraction intensities of a protein crystal as the accumulated doses increase. The consequence of damage is a preferential loss of the high-resolution information. Radiation damage is a dose-dependent process and cannot be reduced by adjusting the dose rate (flux) of the irradiating electrons. The strategy used to minimize the damage is to record the diffraction or image data from a specimen area that has not been previously exposed to electrons for purposes of focusing or other adjustments (Unwin & Henderson, 1975). This is called a minimal or low-dose procedure. In addition, keeping a specimen at low temperature (<113 K) allows it to tolerate a higher radiation dose (by a factor of about 4 to 6) before reaching the same extent of damage as at room temperature (Hayward & Glaeser, 1979). It has been shown that damage reduction is minimal below liquid-nitrogen temperature (Chiu *et al.*, 1981). However, there have been some impressive results using the electron cryomicroscope to study membrane protein crystals kept at liquid-helium temperature (4 K) (Kühlbrandt *et al.*, 1994; Kimura *et al.*, 1997; Miyazawa *et al.*, 1999).

### 19.2.3.3. *Other technical factors*

In order to record a three-dimensional data set, the crystals have to be tilted to different angles with respect to the direction of the electron beam. In a typical electron microscope, the highest angle to which the specimen stage can be tilted is about ±60°. Consequently, there is a missing set of data beyond the highest tilt angle, which corresponds to no more than 15% of the entire three-dimensional volume. Because of the radiation damage, a single diffraction pattern or a single image per crystal is usually recorded (Henderson & Unwin, 1975). The quality of a crystal is easily judged by its electron diffraction pattern as captured from a CCD camera during data collection. Evaluating the ultimate quality of images, however, takes more time and requires extensive computational analysis.

There are two major technical problems that often limit the data quality, even though a crystal is highly ordered (Henderson & Glaeser, 1985). One is the flatness of the crystal, and the other is the beam-induced movement or charging of the crystal. The effects of both problems become more prominent when the crystals are tilted to high angles. These effects tend to blur the diffraction spots, resulting in loss of high-resolution data (Brink, Sherman *et al.*, 1998). There are many ways to overcome these technical handicaps. For instance, the type of microscope grid chosen or the method of making the carbon support film is critical for reducing the wrinkling of the crystals (Butt *et al.*, 1991; Glaeser, 1992; Booy & Pawley, 1993). The use of a carbon film, which is a good conducting material, to support the protein crystal appears to reduce specimen charging (Brink, Gross *et al.*, 1998). It has been suggested that using a gold-plated objective aperture is effective in reducing specimen charging by generating a stream of secondary electrons to neutralize the positive charges that have built up on the specimen, which thus acts like an aberration-inducing electrostatic lens. Empirically, irradiating the microscope grid before depositing the specimen also reduces the charging (Miyazawa *et al.*, 1999). All these technical problems that can hamper progress in the completion of the structure determination have gradually been identified and resolved. However, more convenient and more robust experimental procedures for reducing these effects further are desirable in order to enhance the efficiency of data collection.

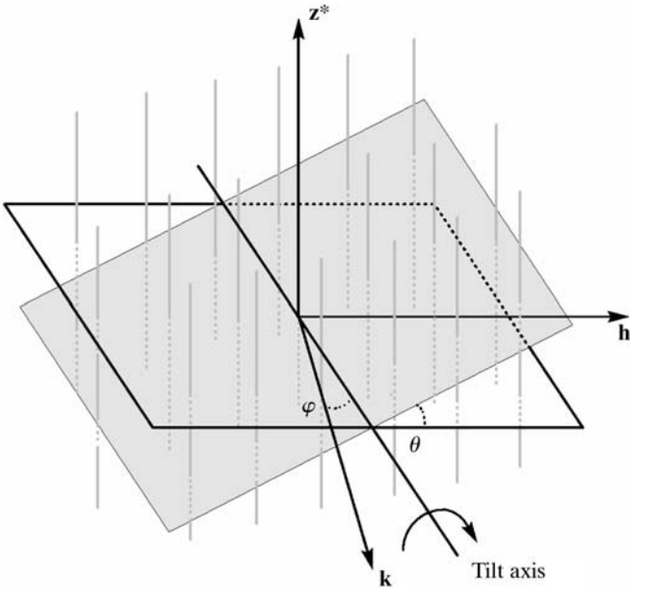


Fig. 19.2.4.1. Schematic diagram of data distribution in Fourier space for a two-dimensional crystal. Both the amplitudes and phases of the structure factors are distributed along the lattice lines passing through the (*h*, *k*) projection plane. The sectional plane shown denotes data from a crystal tilted at a certain angle ($\varphi$), and its tilt axis has an inclined angle ($\theta$) with respect to one of its crystallographic axes. (Courtesy of Dr Jaap Brink at Baylor College of Medicine.)





references