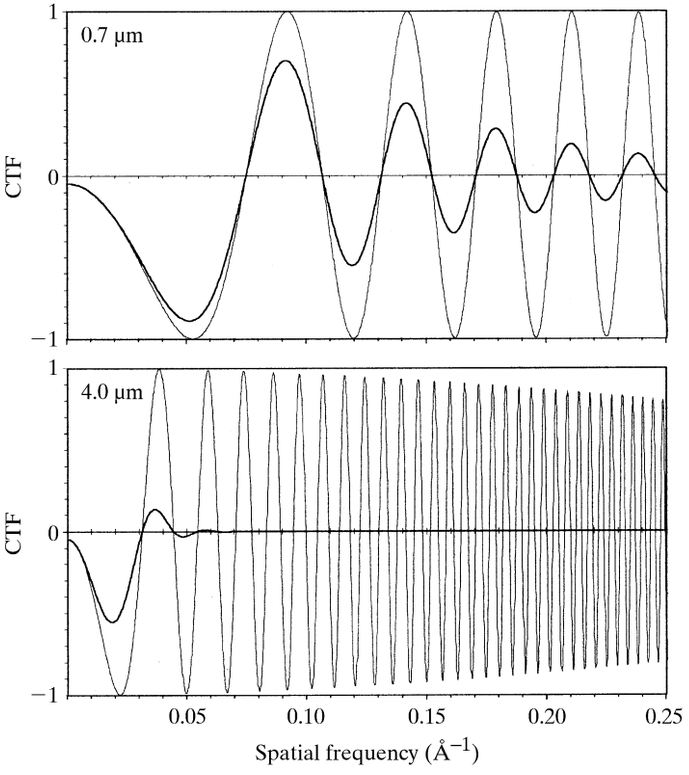

Fig. 19.6.4.4. Representative plots of the microscope contrast-transfer function (CTF) as a function of spatial frequency, for two different defocus settings (0.7 and 4.0 μm underfocus) and for a field-emission (light curve) or tungsten (dark curve) electron source. All plots correspond to electron images formed in an electron microscope operated at 200 kV with objective-lens aberration coefficients $C_s = C_c = 2.0$ mm and assuming amplitude contrast of 4.8% (Toyoshima *et al.*, 1993). The spatial coherence, which is related to the electron source size and expressed as β, the half-angle of illumination, for tungsten and FEG electron sources was fixed at 0.3 and 0.015 mrad, respectively. Likewise, the temporal coherence (expressed as ΔE, the energy spread) was fixed at 1.6 and 0.5 eV for tungsten and FEG sources. The combined effects of the poorer spatial and temporal coherence of the tungsten source leads to a significant dampening, and hence loss of contrast, of the CTF at progressively higher resolutions compared to that observed in FEG-equipped microscopes. The greater number of contrast reversals with higher defocus arises because of the greater out-of-focus phase shifts as described in Section 19.6.4.4.

or uncoated vitrified specimens (Brink *et al.*, 1998) and reduced phase shifts associated with beam tilt.

Images are recorded on photographic film or on a CCD camera with either flood-beam or spot-scan procedures. Film, with its advantages of low cost, large field of view and high resolution (∼10 μm), has remained the primary image-recording medium for most cryo EM applications, despite disadvantages of high background fog and need for chemical development and digitization. CCD cameras provide image data directly in digital form and with very low background noise, but suffer from higher cost, limited field of view, limited spatial resolution caused by poor point spread characteristics and a fixed pixel size (24 μm). They are useful, for example, for precise focusing and adjustment of astigmatism (*e.g.* Krivanek & Mooney, 1993; Sherman *et al.*, 1996). With conventional flood-beam imaging, the electron beam (generally >2–5 μm diameter) illuminates an area of specimen that exceeds what is recorded in the micrograph. In spot-scan imaging, which decreases the beam-induced specimen drift often seen in flood illumination, a 2000 Å or smaller diameter beam is scanned across the specimen in a square or hexagonal pattern while the image is recorded (Downing, 1991). This method is beneficial in the examination of 2D crystalline specimens at near-atomic resolutions (Henderson *et al.*, 1990; Nogales *et al.*, 1998) and has also been used to study some icosahedral viruses (*e.g.* Zhou *et al.*, 1994; Zhao *et al.*, 1995).

For studies in which specimens must be tilted to collect 3D data, such as with 2D crystals, single particles that adopt preferred orientations on the EM grid, or specimens requiring tomography, microscopy is performed in essentially the same way as described above. However, the limited tilt range (±60–70°) of most microscope goniometers can lead to non-isotropic resolution in the 3D reconstructions (the 'missing cone' problem), and tilting generates a constantly varying defocus across the field of view in a direction normal to the tilt axis. The effects caused by this varying defocus level must be corrected in high-resolution applications (Henderson *et al.*, 1990) or they can be partially corrected during spot-scan microscopy if the defocus of the objective lens is varied in proportion to the distance between the beam and tilt axis (Zemlin, 1989).

19.6.4.5. *Selection and preprocessing of digitized images*

Before any image analysis or classification of the molecular images can be done, a certain amount of preliminary checking and normalization is required to ensure there is a reasonable chance that a homogeneous population of molecular images has been obtained. First, good-quality micrographs are selected in which the electron exposure is correct, there is no image drift or blurring, and there is minimal astigmatism and a reasonable amount of defocus to produce good phase contrast. This is usually done by visual examination and optical diffraction.

Once the best pictures have been chosen, the micrographs must be scanned and digitized on a suitable densitometer. The sizes of the steps between digitization of optical density, and the size of the sample aperture over which the optical density is averaged by the densitometer, must be sufficiently small to sample the detail present in the image at fine enough intervals (DeRosier & Moore, 1970). Normally, a circular (or square) sample aperture of diameter (or length of side) equal to the step between digitization is used. This avoids digitizing overlapping points, without missing any of the information recorded in the image. The size of the sample aperture and digitization step depends on the magnification selected and the resolution required. A value of one-quarter to one-third of the required limit of resolution (measured in μm on the emulsion) is normally ideal, since it avoids having too many numbers (and therefore wasting computer resources) without losing anything during the measurement procedure. For a 40000× image, on which a resolution of 10 Å at the specimen is required, a step size of 10 μm [= (1/4)(10 Å × 40000/10000 Å μm^{-1})] would be suitable.

The best area of an image of a helical or 2D crystal specimen can then be boxed off using a soft-edged mask. For images of single particles, a stack of individual particles can be created by selecting out many small areas surrounding each particle. In the later steps of image processing, because the orientation and position of each particle are refined by comparing the amplitudes and phases of their Fourier components, it is important to remove spurious features around the edge of each particle and to make sure the different particle images are on the same scale. This is normally done by masking off a circular area centred on each particle and floating the density so that the average around the perimeter becomes zero (DeRosier & Moore, 1970). The edge of the mask is apodized, which means the application of a soft cosine bell shape to the original densities so they taper towards the background level. Finally, to compensate for variations in the exposure due to ice thickness or electron dose, most workers normalize the stack of

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