A polar Fourier transform (PFT) method is described that facilitates determination and refinement of orientations of individual biological macromolecules imaged with cryoelectron microscopy techniques. A three-dimensional density map serves as a high signal-to-noise model from which a PFT database of different views is generated and against which the PFTs of individual images are correlated. The PFT produces rotation-invariant data particularly well-suited for rapid and accurate determination of orientation parameters. The method relies on accurate knowledge of the center of symmetry and radial scale of both model and image data but is insensitive to the relative contrast and background values of these data. Density maps may be derived from a variety of sources such as computer-generated models, X-ray crystallographic structures, and three-dimensional reconstructions computed from images. The PFT technique has been particularly useful for the analysis of particles with icosahedral symmetry and could be adapted for the analysis of single particles of any symmetry for which a crude model exists or can be produced.
tively stained specimens, but can lead to inconsistent results when applied to low-contrast, noisy images of unstained, vitrified specimens (e.g., Fuller et al., 1995). A statistical method that helps overcome some limitations of this method was developed by Fuller (1987), whose "Modified Common Lines" method is described elsewhere in this issue (Fuller et al., 1996).

Here we describe a Polar Fourier Transform (PFT) method that was developed for use either in conjunction with Modified Common Lines or for de novo determination of particle orientations. The PFT method has been used by us as well as others to determine the structures of several icosahedral viruses (e.g., Dokland and Murialdo, 1993; Cheng et al., 1994a,b, 1995; Hagensee et al., 1994; Kenney et al., 1994; Veniën-Bryan and Fuller, 1994; Wikoff et al., 1994; Fuller et al., 1995; Ilag et al., 1995; Zhao et al., 1995). The method could also be adapted for the analysis of asymmetric macromolecules or those with lower symmetry than an icosahedron. A number of other alignment methods that use reference data in analogous ways to the PFT method have been independently developed (e.g., Harauz and van Heel, 1986; Crowther et al., 1994; Penczek et al., 1994; Radermacher, 1994). A detailed comparison of these and the PFT methods is beyond the scope of this article.

**Fig. 1.** Schematic representation of generation of PFT data for model (a–d) and raw image (e) data. (a) Fifty-two views of an SV40 3D reconstruction (Baker et al., 1988, 1989), obtained by back-projecting the density map in 3° angular increments for orientations covering one-half of the icosahedral asymmetric unit: three-fold view at top left, two-fold at bottom left, and five-fold at bottom right (shaded, half-triangle in inset). Note that 382 views would be obtained if the model was viewed at finer angular intervals (~1°) as is normally done in the initial stage of a PFT refinement. Inset shows the standard convention (Klug and Finch, 1968) in which the icosahedron is aligned with three of its two-fold axes coincident with an x,y,z Cartesian coordinate system. Three angles define the orientation of the view direction: θ (rotation in the xz plane, positive from z toward x), ϕ (rotation in the xy plane, positive from x toward y), and ω (not depicted, defines the rotation of the object about the θ,ϕ view direction). (b) (Left) Two-fold view (θ,ϕ,ω = 90°,0°,0°) of SV40 model; (top right) the same view of SV40 but resampled onto a polar grid (radius, r, increases from bottom to top and azimuthal angle, χ, increases from left to right); (lower right) representation of PFT amplitude distribution (r increases from bottom to top and angular frequency, Δ, increases from left to right). Each row of the PFT is a 1D Fourier transform of the density distribution within the corresponding row (r) of the polar data. (c and d) The same as (b) for three-fold (θ,ϕ,ω = 69.1°,0°,−90°) and five-fold (θ,ϕ,ω = 90°,31.8°,−90°) views of the SV40 model, respectively. (e) The same as (b–d) for a raw image of an SV40 particle viewed close to a five-fold axis (θ,ϕ,ω = 89.9°,−31.4°,20.8°). All panels employ the same color table with brightest colors used to represent highest projected density (images, projections, and polar images and projections) or amplitude (PFT data).
METHODS

Projection and Polar Projection Reference Databases

Analysis of icosahedral particles with the PFT method begins with a 3D model from which m different projected views are generated to produce a reference database (Fig. 1a). For icosahedra, these views cover one-half of the asymmetric unit of the structure (e.g., 1/120th of an icosahedron from θ = 69 to 90° and φ = 0 to 32°). Each Cartesian view (x, y) is interpolated onto a polar grid (r, g), which subdivides the data onto a series of equal-spaced annuli, from r = 0 to a radius just outside the particle edge (Figs. 1b–1e). The width of each annulus is equal to the sampling interval (pixel size) in the Cartesian data and all annuli are sampled the same number of times (r direction) at a spacing fine enough to ensure that no loss of information occurs at high radii (i.e., sampling at least as fine as in the Cartesian image). The resultant polar data consist of the annuli organized in rows (r direction), all sampled the same number of times (g direction). Hence, the polar data are oversampled at small r.

Computation of PFT Database and Initial Origin Determination for Particle Images

Each of the polar projections and images is Fourier transformed along the azimuthal (g) direction to produce a database of PFTs (Figs. 1b–1d) against which the PFT of each “raw” image (Fig. 1e) is correlated according to the scheme depicted in Fig. 2. Thus, the PFT representation is simply a series of one-dimensional (ID) Fourier transforms or rotational power spectra (Crowther and Amos, 1971), one for each annulus of data. This type of representation has the advantage of being rotation invariant (Schatz and van Heel, 1990) which greatly reduces the subsequent number of computations required to identify orientation parameters. Such data reduction leads to successful implementation of the PFT procedure only if an accurate starting estimate of the position of each particle origin known. This origin, where the icosahedral 2-, 3-, and 5-fold symmetry axes meet, is precisely defined for model data, but must be determined for each raw image.

An initial origin estimate (x₀, y₀) for each virus image is obtained by first summing all projected views to produce an averaged projection, then circularly symmetrizing this average about its known center to produce a reference, and finally cross-correlating this reference against each of the n images (Fig. 2, dashed arrows). Experience shows that this procedure is quite reliable with spherical objects, probably because the projected image of a sphere (a circular disk) is centro-symmetric and thus has a well-defined center.

Scaling

The model and image data must be within a few percent of the same magnification for the PFT method to succeed. One way to check the relative scales of the data is to compare ID radial density plots (e.g., Olson and Baker, 1989; Belnap et al., 1993) of the circularly symmetrized reference projection (Fig. 2) and a circularly symmetrized, global average of the images. If necessary, the magnification of the 3D model is adjusted accordingly and the particle image origins are redetermined (Fig. 2, dashed arrows). The absolute contrast and average density of the model and image data need not be carefully scaled because the correlation procedures are insensitive to differences in these characteristics except that the relative contrast of the model and image data must be of the same sign.

Determination of Orientation (θ, φ, ω) and Origin (x, y) Values

Each of the image PFTs is correlated in turn with all model PFTs to identify which model PFT best matches each image PFT (Fig. 2). This process provides an initial estimate of the values of θ and |φ| for each image. The rotation angle, ω, and the sign of

Fig. 2. Schematic diagram of EMPFT procedure. The process typically begins by computing a series of m projections from the 3D model (top row) at 1° angular intervals followed by determination of initial origins of the particle images (dashed arrows). Then, the polar coordinate interpolations, Fourier transformations, and various correlations of the data are performed as indicated (solid arrows), leading to values of θ, φ, ω, x, y for each of the n images. A “best” subset of these images is used to compute a 3D reconstruction (dashed box) and the entire process may be repeated with this new model. Either new particle origins may be determined (dashed arrows) or the newly acquired origin coordinates may be used (thick arrow). Continued cycles of refinement may include the use of 3D models computed from different subsets of images, computation of projections at finer angular increments, and addition of higher resolution data. See text for detailed description of the steps.
Enhanced Refinement with Filtered Data

Refinement is repeated until no further increases in the correlation coefficients can be obtained between model and raw data and an ASCII number of ASCII format data files that list several different correlation coefficients between model and raw data and an ASCII format file that contains the list of $\theta_0, \phi_0, x, y$, and other parameters that serve as a standard input file for many of the icosahedral reconstruction programs (Fuller et al., 1996).

RESULTS AND DISCUSSION

PFT Method

The PFT method relies on the use of a 3D model to screen noisy images of individual particles and refine a set of $\theta, \phi, x, y$ parameters to enable a 3D reconstruction to be computed from the set of images. A major advantage of the PFT method, like that of other model-based methods (e.g., Crowther et al., 1994; Penczek et al., 1994; Rademacher, 1994), is that the raw image data are compared to relatively noise-free, 3D model data rather than other noisy data as is done in the Common Lines (Crowther, 1971) and the Modified Common Lines (Fuller, 1987) techniques. Additionally, the PFT method makes use of all of the available data if so desired, whereas the Common Lines only sample a fraction of Fourier data and this fraction progressively decreases at high resolution (Fig. 3; see also Fuller et al., 1996).

These advantages of the PFT method have led to more consistent results in our analyses of virus images when compared to our use of Common Lines methods. Common Lines often fails or leads to inconsistent results with cryoEM images recorded close to focus (<1.5 $\mu$m) or with viruses that do not have coarse morphological features (see Example 4, below). Our Common Lines analysis of CaMV only succeeded after we recorded focal pairs of vitrified specimens and used a 2.4-$\mu$m underfocus micrograph to determine initial orientations of the particles and applied these values to the same particles in the closer-to-focus (1.2-$\mu$m), lower-dose image (Cheng et al., 1992). As a test, we reexamined the same 1.2-$\mu$m image with Common Lines and were only able to identify the correct orientation view for 1 of the 21 CaMV particle images that went into the original 3D reconstruction. Similarly, the success rate of Common Lines in finding orientations of low pH-treated Semliki Forest viruses was less than 10% (Fuller et al., 1995). We have now used the PFT method to successfully examine a large number of viruses from micrographs recorded at <1.5 $\mu$m defocus (Cheng et al., 1994a,b, 1995; Hagensee et al., 1994; Wikoff et al., 1994; Ilag et al., 1995; Zhao et al., 1995).

The degeneracy of the common lines for particles oriented near one of the icosahedral symmetry axes often makes it very difficult to correctly identify such particles with confidence (Fuller, 1987). Hence,
even though the quality of the images of such particles may be quite good, these data are usually omitted from the computation of the 3D reconstructions. The PFT method appears to have no orientation bias and we have repeatedly found that this method correctly identifies a high percentage of particles that would have been rejected in a Common Lines analysis (data not shown). In addition, the Modified Common Lines and PFT methods are better able than Common Lines to discriminate between the relative handedness of the projection images obtained from different particles (e.g., Conway et al., 1995).

A significant advantage of the PFT method is the relative speed with which the orientations and origins of a large number of particle images can be determined. This efficiency is partly a consequence of greatly reduced computations needed for the PFT method compared to the Common Lines methods (Crowther, 1971; Fuller, 1987), especially when larger size data sets (>50–100 particle images) are analyzed. For PFT, the computations increase linearly with the number of particle images. Common lines and cross-common lines algorithms (Crowther, 1971; Fuller, 1987) on the other hand, require computations that increase in number at a faster rate than the number of images and hence, even the use of massively parallel computers (Martino et al., 1994) may not provide a practical solution to the analysis of very large data sets (>1000 images). A more significant, though less easily quantified, advantage of the PFT method is that it requires much less user intervention compared to the Common Lines methods. The poor success rate in determining good initial estimates of particle orientations with Common Lines necessitates an often tedious process of examining the data for each particle individually and using various criteria to select the most promising particle images for cross-common lines analysis (Fuller, 1987; Fuller et al., 1996).

The Model

The main restriction of the PFT method is the requirement for a “good” starting 3D model. However, the generation of a suitable model is often not a major obstacle in practice. Initial model data can be generated in a variety of ways. The method chosen usually depends on the particular nature of the specimen being examined. We have used density maps from (1) simple, computer-generated, icosahedral models; (2) 3D reconstructions of structures expected to closely resemble the virus under investigation; (3) 3D reconstruction of the virus computed from a single particle image; and (4) an atomic model of the same or similar virus. Thus, the PFT method is analogous in principle to the molecular replacement method (Rossmann, 1972), which is extensively used to aid the phasing of X-ray crystallographic data of viruses and other macromolecules. Several illustrative examples of the use of the PFT method are presented below.

Example 1: Computer-Generated $T = 7$ Model—Analysis of CaMV

For some viruses like the dsDNA animal papovaviruses and the dsDNA plant caulimoviruses, a very simple computer model serves as a useful starting point for the PFT analysis (Figs. 4a–4c) because these viruses have capsids composed of large morphological units (capsomers) arranged on an enantiomorphic, $T = 7$ icosahedral lattice (Caspar and
Klug, 1962). A model with 72 spheres arranged on a $T = 7l$ (left-handed) lattice (Fig. 4a1) and with overall dimensions adjusted to give an effective diameter of ~52 nm was used to initiate refinement of a set of 20 cryoEM images of CaMV. The subsequent models obtained during seven cycles of PFT refinement (Figs. 4a2-4a8) show how they converge to a final and correct solution. The starting model contains only very-low-resolution information (spheres) yet the final reconstruction shows 12 capsomers with pentameric substructure and 60 capsomers with hexameric substructure as was discovered in the original analysis of this structure (Cheng et al., 1992). Correlation coefficients computed in reciprocal (Fig. 4b) and real (Fig. 4c) space demonstrate that the agreement between the raw and model data improves during refinement.

Example 2: Polyoma Model—Analysis of CaMV

Polyoma virus (~50 nm diameter) has 72 pentameric capsomers arranged on a $T = 7d$ lattice (Rayment et al., 1982). Given the close similarities in size and gross morphology of polyoma and CaMV, we used the 2.2-nm resolution X-ray structure of polyoma (Griffith et al., 1992) as a model (Fig. 4d1) to refine the CaMV data set used in Example 1 above. The CaMV pentamer–hexamer structure again emerges despite the potential incorrect bias of the all pentamer polyoma model (Fig. 4d). This result is analogous to experiments in which various incorrect models were used to phase polyoma X-ray data and these all led to the same, correct solution (Rayment et al., 1983). Note that the CaMV reconstruction ends up right-handed ($T = 7d$: Fig. 4d6) like the polyoma model (Fig. 4d1). In Example 1, the CaMV ended up left-handed ($T = 7l$: Fig. 4a8) like the computer model (Fig. 4a1). Thus, the starting model dictates the handedness of the final reconstruction because there is no a priori knowledge of handedness in the projected images themselves. A correct determination of the absolute hand of the reconstruction must be derived from other sources of information such as might be obtained by tilting experiments in the microscope (e.g., Klug and Finch, 1968; Finch, 1972; Cheng et al., 1995) or by comparison with atomic resolution data (e.g., McKenna et al., 1992; Olson et al., 1993; Smith et al., 1993a,b; Cheng et al., 1994b; Porta et al., 1994; Wikoff et al., 1994; Ilag et al., 1995). Nonetheless, despite the handedness ambiguity, the fine details of the structure emerge from the data and not from the model.

Example 3: CPMV Model—Analysis of $\phi X174$ and Vice Versa

A third test of the PFT routines involved the use of 3D reconstruction data for a virus different from the one being examined (Figs. 5a and 5b). CPMV is a ssRNA plant virus with an outer capsid that ranges in diameter from 25.4 to 30.8 nm (Stauffacher et al., 1987) and $\phi X174$ is a ssDNA bacteriophage with an outer capsid that ranges in diameter from 24.7 to 33.0 nm (McKenna et al., 1992; Olson et al., 1992; Ilag et al., 1995). At first glance these two viruses appear quite similar with prominent “caps” of density at the 12 icosahedral vertices. Closer inspection reveals many striking differences in the capsid sub-
structure. Nonetheless, when a 3D reconstruction of CPMV (Porta et al., 1994) was used as a starting reference model for the refinement of φX174 image data (Fig. 5a) or a 3D reconstruction of φX174 (Olson et al., 1992) was used as a starting reference model for the refinement of CPMV image data (Fig. 5b), the

![Diagram](image-url)
image data constrained the PFT refinement to yield final, correct structures.

Example 4: HRV14 and HRV16 Models—Analysis of HRV16

The atomic structures of several of the 114 serotypes of HRV (~30 nm diameter) have been determined (Rossmann, 1994). Despite several attempts, a 3D reconstruction of HRV was never accomplished by use of Common Lines methods (N. H. Olson, unpublished results). Lack of success was attributed to the extremely noisy nature of the HRV images and the overall smooth morphology of the protein capsid. However, a 3D reconstruction of HRV16 complexed with the N-terminal two domains of ICAM-1, the receptor molecule for the major group of HRV, was successfully obtained by means of Common Lines methods (Fig. 5d1; Olson et al., 1993). This worked presumably because the ICAM molecules add significant surface features to the HRV structure. This 3D reconstruction, after the ICAM molecules were computationally trimmed off (Fig. 5d2), succeeded as a starting model for refinement of a data set of native HRV16 images and led to a 3D reconstruction of HRV16 (Fig. 5d3). As a further test, we used the X-ray structure of HRV14 (Fig. 5c2; Rossmann et al., 1985) to compute a 2.5-nm-resolution model to refine the same HRV16 images. A similar 3D reconstruction of HRV16 resulted (Fig. 5c3). Comparison of these two reconstructions (Figs. 5c3 and 5d3) with a low-resolution representation of the HRV16 atomic structure (Fig. 5c1; Oliveria et al., 1993) shows that HRV16 reconstructions indeed show very subtle yet genuine features that distinguish the two HRV serotypes. For example, a ridge of density in HRV16 that crosses the “canyon” surrounding the star-shaped pentamers (Fig. 5c3, arrow) is absent in HRV14 (Fig. 5c2, arrow).

Example 5: Analysis of CPV–Fab Complex

The atomic structures of CPV (25.5 nm diameter; Tsao et al., 1991; Fig. 5e1) and several antibody Fab fragments (Wilson and Stanfield, 1993) are known. In the analysis of the binding of a neutralizing monoclonal antibody to a CPV epitope, two different models were used to obtain 3D reconstructions of the virus–Fab complex from the same set of cryoEM images (Wikoff et al., 1994). One model (Fig. 5e2) was generated by docking the atomic model of Fab(Kol) (Marquart et al., 1980) to the atomic structure of the native CPV (Fig. 5e1, Tsao et al., 1991). The second model was obtained by applying the Common Lines method to a single CPV–Fab particle image to generate a low-resolution, “single-particle” 3D reconstruction (Venién-Bryan and Fuller, 1994) that showed “promising” features (i.e., lumps sticking out of the surface of a ball of density: Fig. 5f1). Both models led to virtually identical 3D reconstructions (Figs. 5e3, 5f2, and 5g). Close inspection of the “pseudo-atomic” model of the complex (Fig. 5e2) in hindsight showed that the Fab(Kol) model was placed in approximately the correct location with respect to the surface of CPV, but was ~60° off in its axial orientation. Thus, the incorrectness of the finer details of the pseudo-atomic model of the complex did not prevent the PFT routine from leading to a reliable 3D reconstruction.

Example 6: FHV X-Ray Model—FHV Analysis

For the spherical viruses whose atomic structures are known, it is mainly the highly ordered protein shell that is revealed. The nucleic acid is generally missing in X-ray maps because the bulk of the genome does not adopt icosahedral symmetry. In addition, X-ray diffraction data are seldom recorded or analyzed at resolutions lower than 1.5–2.0 nm. Nonetheless, the X-ray structure of the protein capsid still serves as an excellent model for PFT refinement of virion images (Fig. 5h). Indeed, the 3D reconstruction of the ssRNA, T = 3 insect virus, FHV (Figs. 5h2 and 5h4: Cheng et al., 1994b), which was obtained by use of the protein portion of the X-ray model (Figs. 5h1 and 5h3: Fisher and Johnson, 1993), shows the distribution of the RNA (Fig. 5h4). A difference map, computed by subtracting the protein X-ray model from the EM map, showed highly ordered regions of RNA (data not shown) that corresponded exactly with the small portion of RNA that was seen in the original X-ray structure (Fisher and Johnson, 1993). Thus, this “control” experiment showed that even subtle, internal features emerge from the analysis of the cryoEM image data despite their absence in the starting model for the PFT analysis.

Example 7: Influence of Model on Results

The 3D reconstruction of the ssRNA, T = 4 insect virus NjβV (~40 nm maximum diameter; Olson et al., 1990) failed as a model for refining a set of FHV images with the PFT routines (Figs. 6a–6d). The reconstruction obtained after five cycles of refinement exhibited features that resembled both viruses (Fig. 6d) but was obviously wrong. Continued refinement led to no significant changes. The precise reasons for the failure are not known, although the magnification of the NjβV reconstruction was carefully rescaled to maximize the fit with the smaller FHV (34 nm maximum diameter: Wery et al., 1994). Apparently, the structure of NjβV differs so significantly from that of FHV that the FHV images correlate poorly with the projected views of the NjβV model. This “bad” model influences the PFT refinement and leads to a final solution that bears no resemblance to the initial model and is also incorrect.
To further test the extent to which the model and image data influence the final results, we generated a data set of shot noise images (Fig. 6f) and refined these images against a simple 3D model. (e) 3D computer model of T particles of polyoma capsid protein. (f) One of a 50-particle data set of “shot-noise” images. (g–h) 3D reconstructions obtained after the first and fourth cycles of PFT refinement, using (e) as starting model.

CONCLUSIONS

The last example serves as strong reminder that not just any model succeeds with the PFT analysis. Our experience with numerous 3D reconstructions clearly demonstrates that, a model which “reasonably” mimics the image data can lead to a reliable 3D reconstruction (Table I). Under these conditions the PFT algorithm appears to be quite robust. If a particular reconstruction seems questionable, one can compute and compare the statistical significance of independent reconstructions (e.g., Milligan and Flicker, 1987), either starting with slightly different models and using the same images, or, if there are sufficient numbers of images, they can be subdivided and processed separately with the same model. The self-consistency of a set of images can also be checked by means of cross-common lines residual tests (Fuller, 1987).

The PFT method is a valuable adjunct to Common Lines procedures (Fuller et al., 1996) because it provides rapid refinement and assessment of the quality of large numbers of images. Use of the PFT method in conjunction with spot-scan imaging and use of a field-emission, intermediate voltage micro-

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* Table lists selected examples of virus structure determinations performed strictly in our lab in which the PFT method was an integral part of the data analysis. These examples illustrate the variety of different models that can be successfully employed in the PFT refinement procedures. "Common Lines" signifies that the starting model for PFT refinement was a preliminary (typically low-resolution or with a limited set of images) 3D reconstruction that was computed with Common Lines (Crowther, 1971) and Modified Common Lines (Fuller, 1987; Fuller et al., 1996) procedures. "Single-particle 3D" signifies that the model for PFT analysis was a low-resolution 3D reconstruction computed from an image of a single particle whose view orientation parameters, (θ, φ, ω), were estimated by the Common Lines method (Veniel-Bryan and Fuller, 1994). "X-ray" signifies that the initial PFT model was derived from the atomic coordinates of a virus whose structure was solved at high resolution by X-ray crystallography. All remaining models were 3D reconstructions computed by Fourier-Bessel techniques (Fuller et al., 1996) of viruses whose structure was believed to be similar to the virus being examined. Although most of these examples are from unpublished studies, the structure determinations of the following viruses have been reported: CCMV<sub>E</sub> (Zhao et al., 1995), CPV-Fab (Wikoff et al., 1994), FHV (Cheng et al., 1994b), HPV<sub>L,1L2</sub> and HPV<sub>L,1</sub> (Hagensee et al., 1994), L-A and UmV (Cheng et al., 1994a), RRV (Cheng et al., 1995), and δX174<sub>UV</sub> (Ilag et al., 1995).
to study other macromolecular structures, including those that are nonspherical and nonsymmetrical, are good but will require that initial particle origins be determined separately and not refined against the average model projection. Subsequent refinement of origins could proceed normally after the initial orientations are determined.

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