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TYSON: Robust searching, sorting, and selecting of single particles in electron micrographs

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Abstract

We here present TYSON, a new program for automatic and semi-automatic particle selection from electron micrographs. TYSON employs a three-step strategy of searching, sorting and selecting single particles. In the first step, TYSON finds the positions of potential particles by one of three different methods: local averaging, template matching or local variance. The practical merits and drawbacks of these methods are discussed. In the second step, these potential particles are automatically sorted according to their probability of being true positives. Many criteria are provided for this sort. In the final -interactive- step, whole categories of poorly fitting false positives can be removed with a single mouse-click. We present results obtained using cryo-EM micrographs of both spherical virus particles and asymmetric particles. The procedures are fast and use of TYSON allowed, for example, some 20 000 particles to be selected in a single working day.
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1. Introduction

Cryo-electron microscopy and single particle image analysis are powerful tools for structural biology. The resolution of three-dimensional reconstructions of macromolecular complexes has improved significantly in recent years (van Heel et al., 2000). However, a major problem in cryo-EM is the low signal-to-noise ratio of images of individual particles as a result of the low doses of electrons that must be used in order to avoid beam-induced radiation damage. High resolution structure determinations can therefore only be achieved by averaging a large number of macromolecular images. Henderson (1995) estimated that a minimum of some 12 000 particles are theoretically needed for achieving near-atomic resolution (higher than 4 Å), a number that in practical analysis may have to be increased to more than one million particles (Glaeser, 1999).

The first step in the process is the selection of particles from the digitized micrographs. This step is performed interactively by visual inspection or by semi-automated methods. Particle selection is currently one of the most labour-intensive steps in the process of structure determination by single particle cryo-EM. A number of methods for automatic particle detection have been proposed (Frank and Wagenknecht, 1984; Harauz and Fong-Lochovsky, 1989; Lata et al., 1995; Ludtke et al., 1999; Ogura and Sato, 2001; Roseman, 2003; Stoschek and Hegerl, 1997; Thuman-Commike and Chiu, 1995; van Heel, 1982). Most of these procedures use the classical cross-correlation approach (Saxton and Frank, 1977) for template matching. The template image approach can be performed using a generic reference image, such as a rotationally averaged reference particle (a "blob"), or using a specific set of known views (projections) of the structure under investigation. Particles in the input image are found by localising peaks in the cross-correlation of the reference and the entire input image. This technique requires substantial computing

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resources for performing the necessary Fast Fourier transforms (FFT) and may be sensitive to background noise. The approach has been used successfully for finding spherical viruses using a generic circular-symmetric blob as a reference image. The generic reference approach is not so well suited for asymmetric particles, which may exhibit significant variations in the images when viewed in different orientations. Currently, methods are being developed which use different projections of a three-dimensional model of the object as a template. These, however, require prior knowledge of the structure and multiple searches must be performed. An extensive review of various particle detection methods is given by Nicholson and Glaeser (2001).

In what probably was the first paper on the issue, van Heel (1982) presented the local variance method for detecting particles in an electron micrograph. This method does not use a model of the particle to locate the objects, but looks for areas with a high local variance. Since this method detects objects irrespective of their shape, it might be well-suited to find asymmetric particles. Like the other approaches discussed, it will also generate false positives, such as aggregates and ice particles (van Heel and Schatz, 2003). Although this method has not yet been used extensively in single particle cryo-EM, it is widely used in X-ray crystallography for establishing the location of protein within a crystal lattice in the case of substantial phase errors (Abrahams and Leslie, 1996).

Our TYSON program provides the user with different methods for detecting single particles including: local averaging, template matching and the local variance operator. Sorting and selecting steps have been implemented in the program allowing for convenient removal of false positives.

2. Methods

As was already mentioned TYSON uses a three-step approach (search, sort, and select) to find particles in electron micrographs. In the first step an initial set of particle coordinates is determined. TYSON uses a minimum of constraints, and as a result false positives will be accepted in the first step of particle picking. The second -interactive- step of the processing allows the clustering of false positives, which are removed in step three.

The methods used in these steps are outlined below.

2.1. Step 1: Search particles

In order to determine the initial set of possible particle coordinates, TYSON offers a choice of three different methods: local averaging, template matching, and the local variance method. Different methods are suitable for various types of particle. The first is well suited to spherical particles with an average intensity higher than the background. Template matching is also well suited for spherical particles. The local variance method is the optimal technique for finding asymmetric objects. The variety of methods available makes TYSON suitable for a wide variety of biological specimens.

In local averaging the total intensity in a disc- or ring shaped area around each point is calculated. This method was described earlier by Kivioja et al. (2000), who implemented it in real space. In TYSON these calculations are performed in Fourier space by calculating the convolution of the image and a binary image of a disc (or ring) valued 1 inside and 0 outside using the convolution theorem. Adjustable parameters are the outside diameter and in the case of a ring area the inside diameter (see also Fig. 1).

After calculation of the locally averaged map, a peak search algorithm locates the initial particle positions. The only constraint in this peak search is the minimum distance between different peaks in order to avoid false negatives as much as possible. By default, this distance is set to the approximate particle size to avoid accepting overlapping particles. Optionally one can limit the number of peaks to be searched, in which case only the highest peaks in a given segment (see Section 3) are selected.

The template-matching technique is a well-known method, that is suited for the detection of spherical particles (Frank and Wagenknecht, 1984; Ludtke et al., 1999; Roseman, 2003; Thuman-Commike and Chiu, 1995; Stoschek and Hegerl, 1997). In this method the cross-correlation between a micrograph and a template image is calculated. Since it is more efficient to perform these calculations in Fourier space, the correlation theorem is applied. The template image can for instance be a spherically averaged image of the particle. TYSON supports a large number of image formats (e.g., JPEG and png, etc.) of the template. After calculation of the correlation map a peak search algorithm with the same constraints as described above is applied to determine the initial set of particle coordinates.

The third method of finding the initial set of coordinates, the local variance method, was first described in (van Heel, 1982). It is based on the fact that areas of a micrograph where objects are present, have a higher local variance than the background due to the contributions of the object's contrast. The local variance calculated for an area A at point $\vec{r}$ is calculated using the formula

$$\mathrm{Var}_A(\vec{r}) = \frac{1}{N}\sum_{n=1}^{N} I_n^2(\vec{r}) - \frac{1}{N^2}\left(\sum_{n=1}^{N} I_n(\vec{r})\right)^2, \tag{1}$$

in which N is the number of pixels inside area A and $I_n(\vec{r})$ is the measured intensity at point $\vec{r}_n$, or as a convolution

$$\mathrm{Var}_A(\vec{r}) = I^2(\vec{r}) \otimes \frac{A(\vec{r})}{N} - \left(I(\vec{r}) \otimes \frac{A(\vec{r})}{N}\right)^2. \tag{2}$$

Again, in order to increase efficiency, the calculations are performed in Fourier space.

Before calculating the variance map, the micrograph is band pass-filtered to remove unwanted frequencies. Particles are located by searching peaks in the local variance map. Since the particles are not always as well aligned as those detected using the methods described above, TYSON can optionally move the peaks to a high value in the low pass-filtered image. This makes use of another detection method also described in (van Heel, 1982), but TYSON it is only used to center the particles in the boxes (the search area is no larger than the particle size). The latter refinement only works when the average density of the objects differs (slightly) from the background. Currently, other methods of alignment are also being implemented in TYSON.

2.2. Step 2: Sort particles

After identifying possible particle positions using the methods described above, the initial set of coordinates still contains a large number of false positives. The second step sorts these coordinates according to the probability that they define a proper particle. To identify false positives, the program no longer operates on the entire micrograph, but instead only uses the boxed areas generated in step one. This allows the efficient use of other, more elaborate methods, such as cross-correlation, on the small scale images. The boxed areas are shown in particle galleries and a variety of criteria are available for the user to automatically sort the particles in this gallery. The criteria include: cross-correlation with respect to the average image; statistic measures; extreme pixel values, and symmetry considerations.

By default, the average of some or all of the boxed areas is calculated and subsequently the cross-correlation coefficient of the individual areas with this average is determined. This coefficient is then used to rank and sort the boxes in the gallery. Additional sorting criteria include the average intensity and standard deviation within a box. The number of pixels above a certain threshold value (average I, average $I + 1\sigma$, etc.) can be used to remove boxes containing dust or ice particles. Symmetry criteria use the amount of 2- or 4-fold symmetry by rotating the particle 180° resp. $3 \times 90°$ and calculating the cross-correlation with respect to the original. We are currently extending the set of sorting criteria employed in TYSON.

2.3. Step 3: Select particles

In the gallery of sorted particles, true particles usually cluster, as do false positives such as fringes or ice- or dust particles. Entire categories of false positives can subsequently be removed by a single click of a mouse button. Steps 2 and 3 may be iterated using different criteria until all false positives are removed. We found this to be considerably more effective than adjusting a number of variables for the entire micrograph to end up with a satisfactory set of coordinates, the strategy followed by most other particle picking programs.

3. Implementation details

TYSON is designed to read entire micrographs, which can be in a number of image formats, including IMAGIC, MRC, and TIFF. Subsequently, the image is divided into smaller segments speeding up the computer memory-intensive calculations for step 1. The segments overlap in order to avoid missing the particles close to segment edges. Segments may be visualized on the micrograph and may be excluded from the initial search by a single click of a mouse button. Thus, TYSON can be prevented to look for particles in unwanted regions containing carbon foil or large aggregates. TYSON estimates the most suitable segment size and overlap size from the magnification of the image and the size of the particles, but these default parameters can be adjusted. Since the calculations in step 1 may take some time to complete for a full micrograph, the operations can also be performed for a large batch of micrographs without any user interaction. In a subsequent run of TYSON the individual input images can be loaded and the particle positions imported in order to directly start working on the more interactive steps 2 and 3. All calculations in step 1 are performed in Fourier space using the fftw-library (Frigo and Johnson, 1998), which is normally considerably faster than real space methods. Note that TYSON can thus also be used to interactively edit (in steps 2 and 3) the automatic particle selection results created by the IMAGIC automated particle selection program (van Heel and Schatz, 2003).

Particle coordinates are indicated on the electron micrograph, allowing the removal of false positives in unwanted regions by clicking or dragging the mouse on that particular area. Positive hits are also displayed in a separate window as a particle gallery, from which they can similarly be removed. However, usually the particles in the gallery are sorted using the criteria described in the previous section, allowing the user to visually determine a threshold below (or above) which particles are removed. Areas below (or above) the threshold can also be marked/unmarked for removal (allowing the user to intervene) prior to actually deleting the unwanted boxes. The final set of particles can be saved in the IMAGIC format or the coordinates can be exported in a ".plt" file for use in IMAGIC (van Heel et al., 1996) or a ".db" file for use in EMAN (Ludtke et al., 1999).

The program is user-friendly, it is menu-driven, and all particle removals can be undone. Other features that improve the usability include a zoom function, which

allows close inspection of the micrographs, image export and print functions for presentations and a help-function describing all the different options in TYSON. It should be noted that although TYSON is designed to pick particles automatically, it also allows the user to pick (additional) particles by hand.

The program was written in C++ using the Windows compatible Qt library to build the graphical interface. TYSON has been tested on both RedHat and Mandrake Linux systems.

4. Results

In order to test the program and to compare the various detection methods three different test specimens were used. The samples were unstained single particles suspended in unsupported ice over holey carbon films. The results obtained for these test specimens are described below.

4.1. Symmetrical particles

In order to test TYSON on symmetric particles, micrographs of bacteriophage MS2 were used (Koning et al., 2003). MS2 has an icosahedral structure ($T = 3$) (Golmohammadi et al., 1993), which is nearly spherical with a diameter of approximately 28 nm. The local averaging method and the template method described above are the obvious choices for locating these particles. For the local averaging method a binary disc of a size similar to that of the particles was used. For the template method the average of 70 particles was used to create the template image. An example of part of a micrograph (1950×1770 pixels) of MS2 particles is presented in Fig. 2. The micrograph was taken on a TECNAI F20 operating at 200 kV. The defocus was approximately -3 μm. The crosses show the result of the initial stage of particle picking using the local averaging method, in which 154 possible particle positions were indentified. Clearly, all particles (except those deemed too close to the edge) were detected. Furthermore, the particles found are already very well centered. The initial set, however, also contained about 50% false positives.

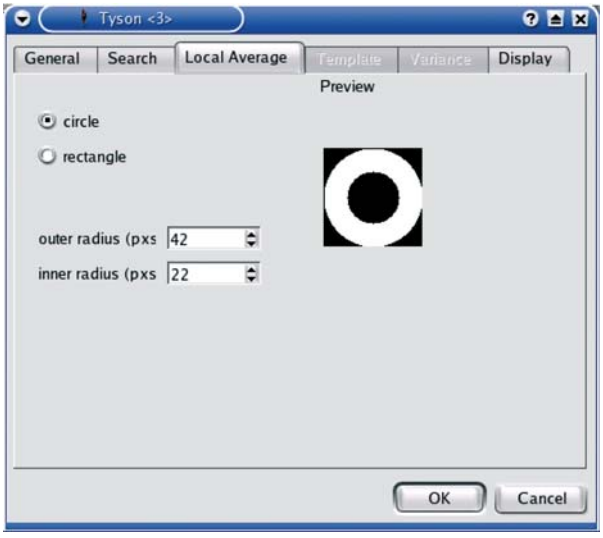

Fig. 1. Setup-window of TYSON showing the adjustable parameters for the local averaging method.

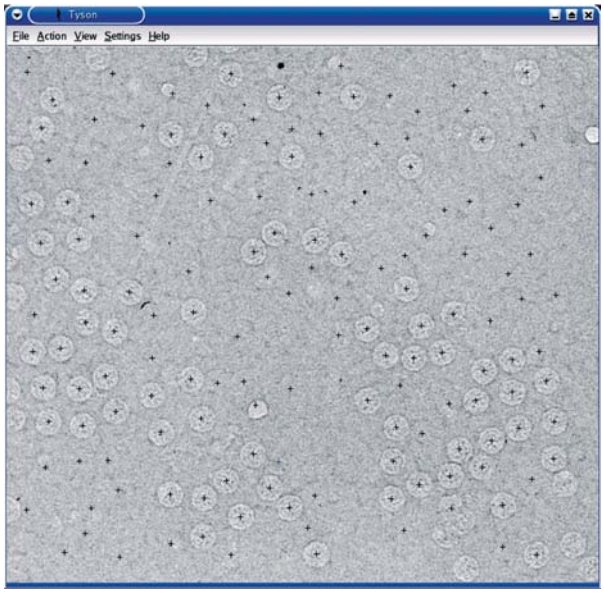

Fig. 2. Part of a micrograph of MS2 particles taken at a magnification of 29 000. The crosses show the initial coordinates determined in step 1 of the particle picking using a binary circular template.

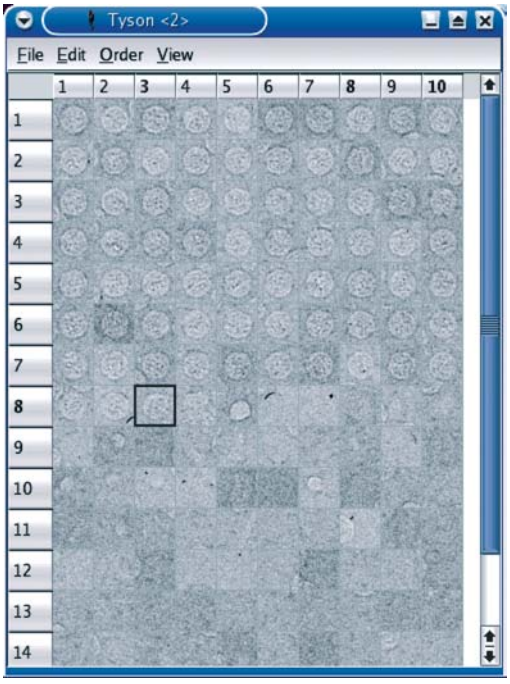

Fig. 3. Gallery window showing the particles found in step 1 sorted using the cross-correlation with the average.

In the case of (nearly) spherical particles, these can easily be identified in step 2 by sorting on the cross-correlation coefficient with the average of all selected boxes. In the example of Fig. 2, sorting the particles shows a clear threshold (Fig. 3). The boxed particle is clearly the last real MS2 particle, whereas the remaining boxes are false positives allowing their straightforward removal in step three. Using this method 73 particles were obtained.

The results obtained using the template method were very similar to the local averaging method (126 hits in step 1; 73 particles after step 3). The number of false positives is slightly lower, since less false objects are detected in areas where only background noise is present. A similar amount of false positives is detected in areas containing ice or dust particles. The objects are also well centered, which makes the cross-correlation criteria in the second stage a powerful tool to identify the false positives.

The local variance method was also applied to determine the initial set of particle positions. The best results were obtained by using a variance area slightly smaller than the particle size. The method finds about 90% of the particles (136 hits in step 1; 68 particles after step 3). The results after step 1 are presented in Fig. 4. False negatives were mostly found when objects are (almost) touching. In this case the peak in the local variance map is often found on the touching edges of the object. The identification of the false positives is slightly hindered by the fact that the particle centers are less well determined using local variance, causing the average used in the cross-correlation method in step 2 to be less well defined.

When using less crowded micrographs, the number of false positives found in step 1 for all methods can be considerably higher. In this case, sorting using the same criterion can be performed iteratively (three to five times), each time removing the worst correlating part of the boxes (e.g., bottom 20% in the particle gallery). With each removal, the average of all boxes approaches the rotational average of the particle, as more noise contributing to the average is taken out.

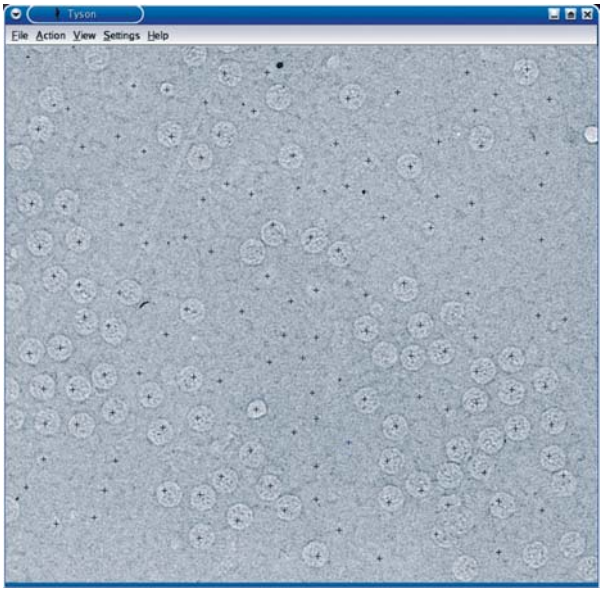

Fig. 4. Part of a micrograph of MS2 particles. The crosses show the initial coordinates determined in step 1 of the particle picking using the local variance method.

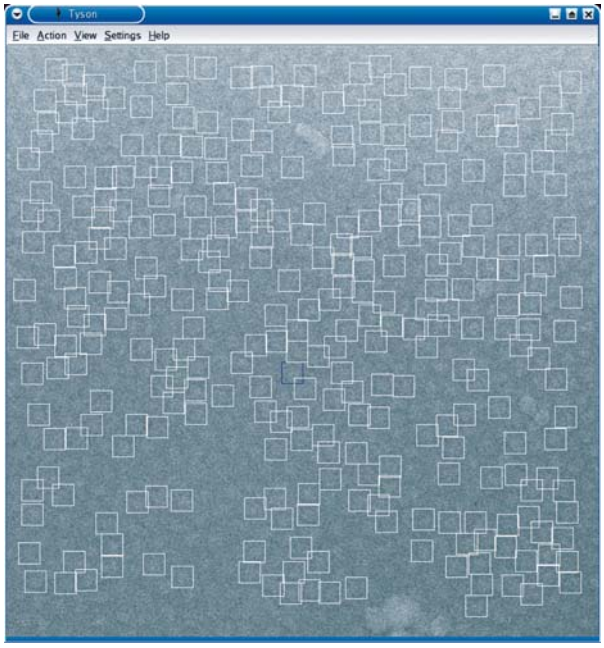

Fig. 5. Part of a micrograph of ribosome 50S subunit particles. The boxes show the result of the particle picking procedure.

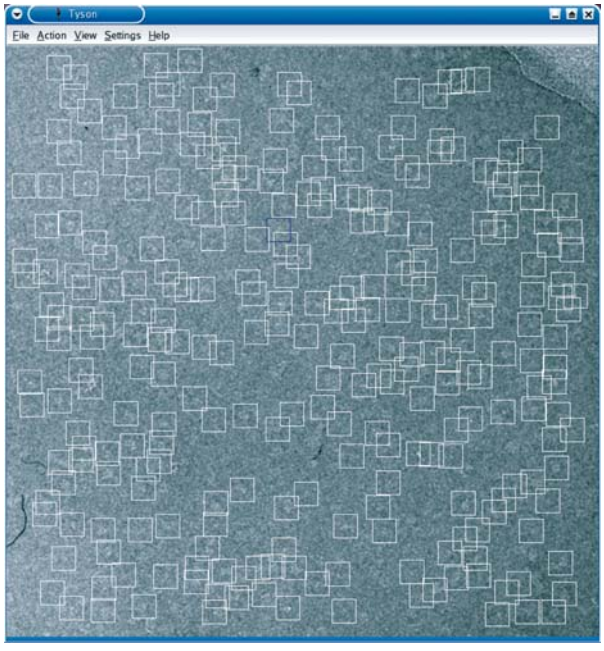

Fig. 6. Part of a micrograph of the RNA polymerase particles. The boxes show the result of the particle picking procedure.

The tests were also performed on spotscan images of the MS2 particles. Using this imaging technique a large number of extra features are introduced. Areas where spots overlap are illuminated twice, whereas other areas are not exposed to the electron beam at all. Furthermore, fringes become clearly visible at the edges of the spots. These areas show a much larger contrast than those parts of the micrograph containing the desired particles. The techniques used in step 1 find large numbers of false positives in these areas, which have a considerable effect on the average of all boxes. Therefore, the boxes were first sorted using their standard deviation as the sorting criterion. This was found to be a very effective method to identify fringes, after which the remainder of the false positives could be removed in a similar fashion as described above.

4.2. Asymmetrical particles

To evaluate the robustness of TYSON in finding asymmetric particles, tests were performed on two specimens: the 50S subunit of *Escherichia coli* ribosome and *E. coli* RNA-polymerase σ54 holoenzyme. Micrographs for both specimens were obtained on a FEG-CM200 microscope operating at 200 kV. For the ribosome particles, both the local averaging method and the local variance method proved very useful. An example of a part of a micrograph showing the ribosome particles picked is shown in Fig. 5. The defocus is approximately −1.1 μm. Clearly, the images contain a large amount of contrast which explains the success of the local average method. In practice step 1 was performed using a local averaging area with a radius of 20 pixels, which is considerably smaller than the particle itself (radius approx. 30 pixels). In the second step the boxed areas (412) were sorted using the standard deviation criterion allowing easy identification of boxed areas containing just ice. A few iterations of steps 2 and 3 using cross-correlation as the sorting criterion removed all false positives resulting in a set containing 312 particles. Using this procedure about 20 000 particles were located in 13 micrographs in less than a working day on an Athlon 2000+ XP pc with 512 MB RAM running Linux.

In micrographs exhibiting lower contrast, the local variance method was found to be the most effective in locating the particles in step 1. Objects were detected by calculating the variance in an area slightly smaller than the particle size. Subsequently the coordinates were shifted to the highest value in the low pass-filtered image within a 50×50 pixel area around the original peak. Again performing a few iterative steps using the cross-correlation criterion proved sufficient for identifying and removing false positives. In both cases the micrographs were pre-processed by applying a 9×9 pixel block filter.

As a second test sample *E. coli* RNA polymerase was used. The dimensions of *Ec* RNA polymerase are approximately $120 \times 150 \times 115$ Å (Finn et al., 2000). A part of a micrograph is shown in Fig. 6 (defocus: −2.9 μm). The red boxes are the result of a particle search using the local variance method. In the case of RNA polymerase, this method was found to be the most effective of all three methods. Particles were searched using a variance area of about the size of the particle (radius approximately 20 pixels). No subsequent alignment was performed after determining the coordinates. In step 2 the particles (798) were sorted using the cross-correlation criterion. About 60% of the particles were removed in two iterative steps. This led to 279 final coordinates as shown in Fig. 6. Comparison of the coordinates with a prior, hand picked set of coordinates, showed that 90% of the particles picked by hand were also picked by TYSON. Furthermore, approximately half as many additional particles were identified. For these micrographs, the success rate of the local variance method was significantly higher than that of the two other methods described here.

5. Discussion and conclusions

Objective, quantative evaluation of the results of a particle detection method remains difficult, as is also described in many other papers dealing with this issue. The absence of a common test set of micrographs of different biological specimens makes it nearly impossible to adequately compare results. Furthermore, since most programs contain some interactive parts, like adjusting parameters using slides or the removal of false positives in step three of TYSON, the results obtained contain subjective elements. As a consequence, presentations of results in terms of percentages of false positives or negatives are somewhat misleading.

Nevertheless, the test cases presented in this paper clearly show that TYSON can successfully be applied to a number of different types of specimens. For both spherical and asymmetric particles, excellent results were obtained with a minimum amount of time spent on interactive computer work. This is achieved by reducing the number of false negatives in the search step at the cost of slightly increasing the number of false positives, and facilitating as much as possible the identification and removal of the latter in the sort and select steps. Usually, in the latter steps, the user spends less than 5–10 min picking particles from an entire micrograph.

The implementation of different search algorithms in step 1 of TYSON makes the program applicable to a wide variety of biological samples. From the results it is clear that for spherical and high contrast particles, the local average or the template method are currently the most useful techniques for locating the objects. The local variance method does find most of the objects, but they are not as well aligned compared to the results of the

other two techniques. As a result the cross-correlation criterion in step 2 is somewhat less powerful for identifying the false positives, a criterion found to be a very powerful tool for spherical particles. Usually, only one or two sort and select cycles are necessary to completely remove all false positives from the set of coordinates found in step 1. Other criteria provided are mainly useful for quickly identifying unwanted objects such as ice or dust particles. It should be noted that with decreasing contrast (e.g., micrographs taken closer to focus) the local averaging method becomes less successful. This is clearly not the case for the local variance algorithm. The latter method may, therefore, prove useful when processing close to focus images. By implementing routines for automatic centering of particles, we anticipate further improving the success rate of the local variance criterion in the particle search.

For asymmetric particles a somewhat different approach appears to be more adequate. When the images have a high contrast the local averaging method can in some cases, as shown by the ribosome example, still be useful. However, when images are taken closer to focus, the local variance method gives the best results. In the case of RNA polymerase this is even true for higher contrast images, presumably because the absence of aggregation allowed the particles to be better centered. Also for asymmetric particles the cross-correlation criterion is the most powerful tool to identify the false positives in step 2. Comparison with hand picked particles indicated that TYSON finds additional ones without a significant number of false negatives. The local variance method is less effective when particles are very closely spaced, since it tends to find the highest values on the connecting edges. However, it is questionable whether one would like to use those particles in the reconstruction. Since the local variance method does not use a model of the particle, bias towards a selection of the possible orientations is avoided. Some other methods like template matching or learning methods using neural networks (Ogura and Sato, 2001) require the user to select a number of particles to construct a template or to use as a learning set, possibly introducing bias.

The template method as well as the local averaging method have been described before. In TYSON the latter, however, is implemented in Fourier space, which considerably increases the speed. The local variance method was proposed earlier (van Heel, 1982), and the results in this paper show this method to be very useful in practice. In the case of asymmetric particles or close to focus images it is the most powerful method of those implemented. The method also detects false positives, but using various sorting criteria, these can easily be removed.

Unlike other programs, TYSON separates the initial coordinates search from the identification and subsequent removal of false positives. In the latter steps TYSON only works with the boxed areas, identified in step 1, allowing particle selection based on various different methods such as cross-correlation or symmetry checks. This is significantly different from most methods, that mainly allow the user to change parameters involved in the peak search, such as peak height thresholds. The TYSON method still requires some user intervention, which is not necessarily a bad thing. However, the interactive work is significantly reduced by providing the various sorting criteria in the particle gallery. Thresholds of false positives can differ significantly for different micrographs, depending on the concentration of the particles and the amount of noise in the images. Therefore, for the moment, user interaction is required in deciding thresholds for false positives removal. Especially the cross-correlation with the average of the boxes identified in step 1 was found to be a very powerful tool for quickly identifying the false positives. In the future we plan to combine the criteria in the sorting stage with clustering algorithms. By implementing various search algorithms and facilitating the identification and removal of false positives, TYSON is a new and powerful tool in the particle picking procedure for three dimensional reconstruction of single particles.

TYSON is freely available from http://www.bfsc.leidenuniv.nl/.

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