Abstract—This paper describes and compares two algorithms for automated recognition of the double-helix point spread function (DH-PSF) within fluorescence microscope images. The DH-PSF is an optical technique for encoding the three-dimensional position of a point emitter with an intensity distribution that is shaped like a double helix in 3D space. The two automated algorithms are based upon template-matching techniques. One algorithm finds single peaks within an image and then matches pairs of peaks together to extract the DH-PSF. The other algorithm matches instances of various rotated forms of the DH-PSF directly using phase correlation. The algorithms are then used to localize blinking fluorescent proteins labeling DNA inside bacterial cells in three-dimensions with 19 nm precision in $x$ and $y$ and 34 nm precision in $z$.

Index Terms—feature detection, image matching, superresolution fluorescence microscopy, three-dimensional localization

I. INTRODUCTION

SUPERRESOLUTION microscopy is a powerful tool for resolving the position of fluorescent molecules beyond the optical diffraction limit of $\sim \lambda/2NA$ ($\sim 250$ nm for optical wavelengths). Methods based on stochastic sampling of molecules, such as photoactivated localization microscopy (PALM/FPALM) [1, 2] and stochastic optical reconstruction microscopy (STORM) [3], create their superresolution images by imaging different sparse subsets of molecules sequentially frame by frame. These subsets must be sparse enough such that the image of each individual molecule is well separated from the image of every other single molecule on an imaging camera. These images can then be fit to a model of the point spread function (PSF) of the microscope to determine the lateral ($x$-$y$) position of each molecule to a precision that is much better than the size of the diffraction-limited spot, thus achieving superlocalization. Once the location of each molecule has been determined, these locations can be plotted altogether to create a computational reconstruction of the structure labeled by the fluorescent molecules, sans any blurring caused by diffraction. Since fluorescence microscopy is noninvasive, demonstrates remarkable label specificity, and produces images with a high signal-to-background ratio, fluorescence microscopy is heavily utilized in cell biology studies. Moreover, superresolution fluorescence microscopy has the potential to allow scientists to observe dynamic processes within live cells that are impossible to observe with a conventional microscope.

The conventional PSF of a microscope is ill suited for three-dimensional localization [4, 5]; thus, in this paper, we create three-dimensional superresolution images from a microscope exhibiting a double-helix point spread function [6, 7]. For each single molecule imaged by a microscope, the DH-PSF creates two Gaussian-like spots. These two spots rotate around their midpoint as a function of the axial ($z$) position of the emitter. Thus, the midpoint between the two spots gives the $x$-$y$ position of the molecule, and the angle between the two spots yields the $z$ position. The relationship of angle versus $z$ position for the DH-PSF is shown in Fig. 1.

Significant image processing is required to process the data from a PALM experiment that uses the DH-PSF, which is termed DH-PALM. In each frame of a movie captured by an imaging camera, the various rotated forms of the DH-PSF must be recognized, isolated, and fitted to a model of the DH-PSF.
PSF to extract the midpoint between the two spots and the angle between the two spots. In this paper, we describe and demonstrate two template-matching algorithms designed to recognize and extract three-dimensional localization data collected from DH-PALM experiments. We also compare the performance of the two algorithms. The two algorithms are then used to determine three-dimensional localizations of blinking fluorescent proteins that are attached to DNA within fixed bacterial cells. We therefore demonstrate that our template-matching algorithms can efficiently extract three-dimensional localization data from single molecule experiments with high precision.

II. PRIOR AND RELATED WORK

Extracting data from a typical PALM experiment can take up to four hours [8] using a Gaussian model of the standard PSF [9]. Thus, image processing is a significant bottleneck when performing PALM experiments. Recently, several algorithms for real-time data analysis have been demonstrated [10-12]. The aforementioned techniques are limited to extracting localization information from single asymmetric Gaussian spots; thus, none of these techniques can be directly applied toward extracting data from DH-PSF experiments. Furthermore, none of these algorithms have applied template-matching methods from computer vision to this recognition and extraction problem. Therefore, we explore the use of linear, shift-invariant, template-matching filters in the following sections.

III. DESCRIPTION OF THE ALGORITHMS

A. Overview of Template Matching

Template matching is a process in which we try to find small, known patches in an image by selecting locations \((p, q)\) that minimize the mean squared error quantity

\[ E(p, q) = \sum_{x=-\infty}^{\infty} \sum_{y=-\infty}^{\infty} [s(x, y) - t(x - p, y - q)]^2. \tag{1} \]

Template matching may be rephrased as a very closely related problem, the maximization of the area correlation,

\[ r(p, q) = s(p, q) \ast t(-p, -q). \]

Using the Fourier transform, maxima of this convolution can be found extremely quickly.

Because of the predictability of our signal, template matching is an excellent choice for the detection of the DH-PSF. We investigated two approaches: single-peak detection followed by a matching step to find the double-helix, and a direct, double-peak detection with phase correlation. The single-peak detection assumes little \textit{a priori} knowledge of the DH-PSF besides the fact that the two lobes are roughly Gaussian and that there is a certain distance range between the two lobes (e.g., they must be at least 2 but no more than 8 pixels apart). The double-peak detection algorithm, on the other hand, uses a database of DH-PSF images when searching input images for template matches, thereby leveraging the known shape and rotation behavior of the DH-PSF.

B. Single-Peak Detection and Pair-wise Matching

The major steps in the single-peak algorithm are background subtraction, peak detection with a Gaussian template, and matching. The main steps of the algorithm are summarized in Fig. 2.

Background Subtraction

From our \textit{a priori} physical knowledge, it is tempting to assume that the background is a constant throughout the image and that it varies slowly with time. A close examination of our data suggests that this is not always the case. The cells contain higher concentrations of fluorescent molecules than the surrounding solution, and this will show up as a false signal in our fluorescence images. In order to cleanly subtract the background, we must explicitly model this spatial variance. We use the fact that the background is slowly changing, but the fluorescent reporter molecules typically only emit for one or two frames. Thus, we take a 60-frame temporal average around the frame of interest and use this as an estimate of the background. We then apply a small Gaussian blur to the background image to improve matching performance. This process may misinterpret some signal photons as background photons, but because of the extent of the averaging this is not an issue. After background subtraction, our image is

![Fig. 2. The major steps of the single-peak detection algorithm. (a) The input image. (b) The image after background subtraction and convolution with a Gaussian. Local maxima of this image that exceed some threshold are selected as peaks. (c) The peak matching step. Annuli are drawn about each peak, and we search for valid handshakes. Three valid handshakes are shown on the top half of the image. The green arrow points to a conflict, where there is too much ambiguity to cleanly resolve, so no matching is done. Similarly, the lone match on the right side is discarded. (d) The final reconstructed DH-PSF. Out of the three DH-PSFs that were matched in the previous step, the Gaussian fitting process only worked cleanly on a single pair, and the other two are discarded.](image-url)
nominally zero mean.

**Peak Detection**

We convolve this image with our template, which we choose to be a Gaussian with a standard deviation of 1 pixel. The design choice of a $\sigma = 1$ pixel is unusual because the actual spots of the DH-PSF have a width of $\sigma \approx 1.25$ pixels. However, because the two lobes of the DH-PSF can be fairly close and because of optical aberrations, using $\sigma \approx 1.25$ pixels will often transform the DH-PSF into a single-peaked blob rather than the desired double-peaked signal. For this reason we sacrifice some SNR and choose to detect a smaller Gaussian than our actual target. The result of this convolution is shown in Fig. 2b.

We then detect the local maxima of this image and restrict ourselves to maxima that exceed some threshold. It is possible to build a theoretical, statistical interpretation into the choice of threshold; the background should be zero mean and photon shot (Poisson) noise limited because of the low signal conditions. We would choose our threshold so that the erroneous detection of a peak in the background is statistically unlikely (say, $p < 0.01\%$). In practice there is enough imperfection in the data from the fluorescence of the bulk cellular mass that we would like to push the threshold out even further than that the statistics would suggest.

**Peak Matching**

After the peaks are identified, they are matched together. An annulus mask with inner radius two pixels and outer radius eight pixels is drawn about each peak (Fig. 2c). For two peaks to be identified as a pair, they must satisfy a handshake criterion: they must contain only each other (no other peaks) in their annulus mask. The restriction of having no other peaks in the annulus helps us to circumvent the ambiguous case where multiple peaks are present in a small radius, and it is not possible to be certain how the pairing should be resolved. Typically, it is possible for a human to make a judgment call and resolve the pairing by experience. We chose not to emulate this behavior algorithmically, simply because a skipped localization (false negative) is more desirable than an incorrect localization (false positive).

Peaks that are matched are then fed into a fitting routine (see section III.D below). The best-fit double Gaussian model corresponding to the input data is shown in Fig. 2d.

**C. Double-Peak Detection with Phase Correlation**

The double-peak detection algorithm uses several templates representing various rotated forms of the DH-PSF for template matching. For each frame, each template is phase correlated with the image, the correlations are combined and peaks are found in the combined correlated image, and these peaks are validated to filter out extraneous matches. These steps are summarized in Fig. 3.

**Template Selection**

The DH-PSF is a complex shape that changes slightly as a function of wavelength. Thus, the templates used in the double-peak detection algorithm are taken from calibration movies measured at the same experimental conditions as the fluorescence data to be analyzed. Six templates were chosen, with the DH-PSF rotated approximately 30 degrees between each template (see Fig. 3a). This represented a good balance between template accuracy and computational speed, since the computational time scales linearly with the number of templates used.

**Phase Correlation**

Template matching with each of the templates is performed using phase correlation in the Fourier domain. Performing the correlation in the Fourier domain can be faster than convolution in real space for large images [13]. Furthermore, phase correlation normalizes the input image and template image amplitudes in the Fourier domain, thereby minimizing the bias of template matching to bright regions of the image. The phase correlation image $r_k$ is computed from the input image $s$ and template $t_k$ with the formula

$$r_k(x, y) = \mathcal{F}^{-1}\left\{\frac{\mathcal{F}(h)\mathcal{F}(s)\mathcal{F}(t_k)^*}{|\mathcal{F}(s)| |\mathcal{F}(t_k)|}\right\},$$

where $h$ is the template, $s$ is the input image, and $t_k$ is the $k$th template. $\mathcal{F}$ denotes the Fourier transform and $\mathcal{F}^{-1}$ denotes the inverse Fourier transform.

---

Fig. 3. Summary of the double-peak detection algorithm. (a) The set of six templates used for phase correlation. (b) The input fluorescence image. (c) The combined output of the phase correlation of all six templates with the input image. (d) Peaks in (c) above a threshold are circled in blue. The six templates can generate multiple “ghost” matches for very bright occurrences of the DH-PSF in the input image. Ghost images are eliminated using a filter, and the validated template matches are shown as green pluses. (e) The final reconstructed image of the DH-PSF's found by the double-peak detection algorithm. There are no false positives shown in this image, while two false negatives occur near the bottom of the input image. These false negatives were found by the template-matching section of this algorithm, but the double Gaussian fitting process failed for these two matches.
where $\mathcal{F}$ denotes the two-dimensional Fourier transform, $\mathcal{F}^{-1}$
denotes the two-dimensional inverse Fourier transform, and
the prefilter $h$ is a Gaussian lowpass filter with a width
$\sigma = 1.5$ pixels. Phase correlation emphasizes high frequency
components in both the input image and the template [13], and
these spatial frequencies typically have a lower signal to
background ratio. The Gaussian lowpass filter emphasizes
lower spatial frequencies present in both the input image and
the template and thus, leads to more reliable estimates.

Local maxima (i.e. peaks) above a threshold are detected in
each image $r_k$, and the locations and magnitudes of each peak
are stored in memory. The width of the Gaussian prefilter and
the value of the peak threshold were chosen empirically to
minimize false positive matches (matches to images that are
not the DH-PSF) and false negative matches (failure to match
to an image of the DH-PSF) simultaneously. In general, the
optimal value of these parameters can be derived if the
additive noise in the original image is modeled with a simple
noise distribution. However, the biological cell data used in
this experiment is complex, and modeling the noise
distribution in the test data is beyond the scope of this work.

**Filtering of ExTRANeous Matches**

Since the peak threshold is set low enough to detect weak
single molecules, extraneous matches occur for stronger
fluorescent signals. If the candidate DH-PSF is bright enough,
the template will match where only one of the spots in the
template overlaps with only one of the spots in the input.
These “ghost” matches (see blue circles in Fig. 3d) are filtered
out by enforcing a minimum distance between all of the
template matches in a given frame of the input data. This
minimum distance is chosen to be the typical diameter of the
DH-PSF image (1.2 μm). If multiple template matches occur
too close to one another, the strongest match is chosen, and the
others are discarded. The template matches that satisfy this
filter are validated template matches (see green pluses in Fig.
3d). These validated matches are then fit to a double Gaussian
model of the DH-PSF (see section III.D below). The best fits
of these validated matches to the model are shown in Fig. 3e.

**D. Converting the Output of Template Matching Filters to
Three-Dimensional Locations**

Once the template matches are found in a given input
image, they are input to the MATLAB optimization function
lsqnonlin, which minimizes the mean-square error
between an eight-parameter double-Gaussian model of the
DH-PSF and the input data. Four parameters are used for each
Gaussian function: amplitude, $x$-center location, $y$-center
location, and width. While this double Gaussian model is only
an approximation of the true shape of the DH-PSF [14], it
represents a good compromise between fitting accuracy and
computational complexity. These fits are then filtered to
ensure that they match the data sufficiently accurately and
produce reasonable reconstructions of the DH-PSF.

The fitting routine produces estimates of the $x$-center
location and $y$-center location of each Gaussian; the midpoint
and angle between the two fitted Gaussians is then calculated.

The midpoint yields the $x$ and $y$ location of the localized single
molecule, while the angle can be converted into a $z$ position
using a calibration curve (see Fig. 1). The precision of the
localizations can be estimated from measuring the number of
photons contained within each image of the DH-PSF [7].

Bright fluorescent beads captured in the fluorescence
images serve as fiduciary markers. These markers are
separately tracked throughout a fluorescence movie to
measure the drift of the microscope stage. These movements
are subtracted from the three-dimensional localizations of
single molecules in order to remove thermal and mechanical
motion artifacts from the superresolution images.

**IV. Experimental Results**

The algorithms were used to process 2760 images of single
blinking fluorescent proteins labeling DNA within a fixed
bacterial cell. The frames were captured using an electron-
multiplying charge-coupled device (EMCCD) camera set to an
exposure time of 15 ms and a gain of 300. These data
represent difficult and complex images for the automated
algorithms to process; the cells themselves contain
background fluorescence, and the fluorescent proteins blink
stochastically.

**A. Error Analysis of the Automated Algorithms**

From the 2760 images in our dataset, we selected 76 high-
activity fluorescence images containing an average of 3
molecules per frame. These frames presented a variety of DH-
PSFs at various signal to background ratios. The localizations
computed by both algorithms were compared against the
localizations extracted by a human, who found each molecule
visually by hand. These localizations are thus a “ground
truth” dataset, against which the performance of the automated
algorithms can be compared. While the human took ~45
minutes to find all instances of the DH-PSF in the test images,
both the single-peak and double-peak detection algorithms
localized molecules at a rate of 15-20 molecules per second.
The performance of the algorithms can be improved by
optimizing lsqnonlin, which used more than half of the
computations for both algorithms.

A summary of the errors made by each algorithm is shown
in Table I. The human found a total of 232 single molecules
in the test data, compared to 180 for the single-peak algorithm
and 197 for the double-peak algorithm. For both algorithms,
93 percent of the matches correspond to molecules in the
human dataset; therefore, the false positive rate for both
algorithms is small and almost identical. However, the single-
peak algorithm extracted fewer molecules than the double-

<table>
<thead>
<tr>
<th>Error Analysis</th>
<th>Human</th>
<th>Single-peak</th>
<th>Double-peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of molecules found</td>
<td>232</td>
<td>180</td>
<td>197</td>
</tr>
<tr>
<td>Number of matches with human</td>
<td>–</td>
<td>167 (93%)</td>
<td>184 (93%)</td>
</tr>
<tr>
<td>Number of false positives</td>
<td>–</td>
<td>13 (7.2%)</td>
<td>13 (6.6%)</td>
</tr>
<tr>
<td>Number of false negatives</td>
<td>–</td>
<td>65 (28%)</td>
<td>48 (21%)</td>
</tr>
</tbody>
</table>

This data was collected from 76 test frames.
peak algorithm; there were 65 false negatives for the single-peak algorithm compared to 48 for the double-peak algorithm. False positives correspond to single-molecule localizations that are not truly present in the fluorescence data, while false negatives correspond to single-molecule localizations that were not extracted from the fluorescence data. The effect of false negatives can be reduced by simply processing more data, while the artifacts created by false positives are difficult to remove unless the data is manually examined frame by frame. Thus, our priority was to minimize the number of false positives produced by our algorithms, at the possible expense of raising the number of false negatives. This is evidenced by the small number of false positives and larger number of false negatives for both algorithms in Table I.

An example comparing the localizations made by the 3 DH-PSF extraction methods (manual extraction, the single-peak detection algorithm, and the double-peak detection algorithm) is shown in Fig. 4. All three DH-PSF extraction methods find a majority of the same DH-PSFs, as shown by the white DH-PSFs in the reconstructed images. The slightly larger false negative rate of the single-peak detection algorithm is also evident from the magenta DH-PSFs in the reconstructed images.

B. Superresolution Images of Fluorescently Labeled DNA inside Bacteria

The two automated algorithms were then used to process the entire collection of 2760 images of the aforementioned fluorescently labeled DNA. The three-dimensional locations of the blinking single molecules were extracted and corrected for stage drift. The single-peak detection algorithm extracted the locations of 775 single molecules within the three cells rendered in Fig. 5. The double-peak algorithm extracted 740 molecules in the same region. On average, each fluorescent molecule emitted ~1750 photons with an average background noise of 3.7 photons/pixel/frame. The camera counts were converted to photons in the same manner as previous studies [6, 7]. Using the previously characterized precision of the

![Fig. 4. Two fluorescence images (top and bottom rows) comparing the molecules recognized by 3 extraction methods: manual (human) extraction, the single-peak detection algorithm and the double-peak detection algorithm. The original fluorescence images are shown in the left panels, and the corresponding reconstructions of the extracted DH-PSFs made by each algorithm are shown in the right panels. The reconstructed images are in good agreement with the original fluorescence images.](image)

![Fig. 5. Several renderings of the locations of fluorescently labeled DNA within fixed bacterial cells. Two-dimensional (top-down) superresolution images are shown of the DNA localized by (a) the single-peak extraction algorithm and (b) the double-peak extraction algorithm. These images show the fluorescence data (red) overlaid with white light images of the cells (gray). The insets in (a) and (b) show diffraction-limited representations of the corresponding images. Note that the diffraction-limited images contain much less detail about the locations of the DNA than the corresponding superresolution images. There is good agreement between the locations of the molecules localized by both algorithms. (c) A three-dimensional rendering of the locations of DNA extracted by the double-peak detection algorithm. The “white cloud” surrounding the localizations simulates the cell volume, as sampled by the DNA locations, thereby showing good agreement with the white light images in (a) and (b). Note that using the DH-PSF enables the 3D location of the DNA to be extracted from fluorescence microscope images with high precision. Approximately 1750 photons were collected on average from each localization with a background noise of 3.7 photons/pixel/frame. This corresponds to a localization precision of 19 nm in x and y and 34 nm in z. The scalebars in (a) and (b) and the grid in (c) correspond to 1 µm.](image)
DH-PSF [7], these photon counts correspond to a localization precision of 19.2 nm in \(x\), 18.6 nm in \(y\), and 34.1 nm in \(z\).

The DNA locations extracted by both algorithms are shown in the superresolution images of Fig. 5. In the plots, the brightness of the molecules corresponds to the number of photons measured in the fluorescence images. The width of the molecules in the \(x\), \(y\), and \(z\) directions corresponds to the precision with which that position is known in \(x\), \(y\), and \(z\). In the two-dimensional superresolution images, there is good agreement between the locations of the blinking fluorescent proteins extracted by both algorithms. The corresponding diffraction-limited images show the marked resolution improvement that is characteristic of optical superresolution microscopy. The molecules extracted by the double-peak detection algorithm are also plotted in three-dimensions in Fig. 5c. This image shows a simulated computation of the cell volume, shown as the “white cloud” surrounding each set of localizations, which agrees quite well with the white light images in Fig. 5b. This rendering also shows that the DH-PSF enables the three-dimensional location of the DNA to be extracted from two-dimensional fluorescence images with high precision.

V. CONCLUSION

In this paper, we have shown that template matching can recognize and extract three-dimensional position information from two-dimensional images of the DH-PSF. These techniques have not been applied before in PALM-like experiments. The algorithms are automated and robust to low signal to background conditions in single-molecule fluorescence images of biological cells. Both the single-peak detection algorithm and the double-peak detection algorithm have a small false positive error rate (~7%), while the double-peak algorithm successfully extracts a slightly higher number of DH-PSFs from test images. We have also demonstrated that these automated algorithms can quickly extract the three-dimensional location of fluorescently labeled DNA within bacterial cells. These new algorithms enable high-throughput automated processing of fluorescence data collected in DH-PALM experiments with a small number of artifacts. In the future, these algorithms can be compiled outside of MATLAB or parallelized on a graphics processing unit to enable real-time processing of DH-PALM data.

APPENDIX

M.D. Lew proposed the project. S.S. Hsieh developed and implemented the single-peak detection algorithm; M.D. Lew developed and implemented the double-peak detection algorithm. M.D. Lew constructed the human-extracted dataset of DH-PSF locations, while S.S. Hsieh performed the error analysis on both automated algorithms. Both authors created renderings of the superresolution localizations of DNA within the bacterial cells.

ACKNOWLEDGMENT

The authors thank David Chen, Derek Pang, and Professor Bernd Girod for their helpful instruction during EE 368. They also acknowledge Monica Schwartz and Professor Lucy Shapiro for preparing the bacterial cell samples. The authors also thank R. Piestun and S.R.P. Pavani at the University of Colorado for providing the DH-PSF phase mask. The authors are grateful to Michael Thompson, Dr. Steven Lee, and Professor W. E. Moerner for collecting the fluorescence images and providing expertise on the DH-PSF.

REFERENCES