Extraction of fluorescent cell puncta by adaptive fuzzy segmentation

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ABSTRACT

Motivation: The discrimination and measurement of fluorescent-labeled vesicles using microscopic analysis of fixed cells presents a challenge for biologists interested in quantifying the abundance, size and distribution of such vesicles in normal and abnormal cellular situations. In the specific application reported here, we were interested in quantifying changes to the population of a major organelle, the peroxisome, in cells from normal control patients and from patients with a defect in peroxisome biogenesis. In the latter, peroxisomes are present as larger vesicular structures with a more restricted cytoplasmic distribution. Existing image processing methods for extracting fluorescent cell puncta do not provide useful results and therefore, there is a need to develop some new approaches for dealing with such a task effectively.

Results: We present an effective implementation of the fuzzy c-means algorithm for extracting puncta (spots), representing fluorescent-labeled peroxisomes, which are subject to low contrast. We make use of the quadtree partition to enhance the fuzzy c-means based segmentation and to disregard regions which contain no target objects (peroxisomes) in order to minimize considerable time taken by the iterative process of the fuzzy c-means algorithm. We finally isolate touching peroxisomes by an aspect-ratio criterion. The proposed approach has been applied to extract peroxisomes contained in several sets of color images and the results are superior to those obtained from a number of standard techniques for spot extraction.

Availability: Image data and computer codes written in Matlab are available upon request from the first author.

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INTRODUCTION

There are a number of recently developed methods for the analysis of DNA microarray spots such as the morphology-based method proposed by Angulo and Serra (2003), the combinatorial image analysis by Glasbey and Ghazal (2003) and the adaptive thresholding by Liew et al. (2003). However, these morphological and statistical thresholding methods are only effective for extracting DNA microarray spots having similar sizes and contained in gridded structures. The main reasons for the unsuitable applications of these methods for the segmentation and extraction of biological images in this study is that these images contain very variable (i) spot sizes, (ii) intensity distributions and (iii) backgrounds. Therefore, extraction of these fluorescent cell puncta using these methods will lead to over/under-segmented results. An associated method for spot extraction has been developed by Xu et al. (1999), which is based on double thresholding and contour-based curve fitting to segment the images of skin cancer. This method is suitable for the segmentation of isolated spots whereas the problem we study herein is not restricted to such cases and its curve fitting technique can only approximate the spot areas, that may lead to a considerable error for the quantification of peroxisome abundance.

In this paper, we present a segmentation method based on the fuzzy c-means for dealing with the more challenging application of extracting and measuring cell puncta images that exhibit low contrast and variable size and cellular distribution, including clustering. The specific application used to test this method is an analysis of the population of peroxisomes in human patient cell lines. Previous findings have indicated a change in the size, cytoplasmic distribution and potential clustering of these cellular organelles in different peroxisomal diseases (Chang et al., 1999). We test our proposed segmentation algorithm for extracting cell puncta with real image and compare the results with those obtained by other standard segmentation methods as well as a current medical image analysis software for spot extraction.

IMPLEMENTATION

Cell culture and immunofluorescence microscopy

Skin fibroblast cell lines were cultured in Dulbecco’s modified Eagle’s medium (high glucose), supplemented with 10% fetal
bovine serum (FBS) and 100 mg/ml penicillin–100 µg/ml streptomycin (Gibco BRL). Cells were processed for indirect immunofluorescence as described previously (Maxwell et al., 1999, 2002) and peroxisomes detected using a rabbit antibody to the peroxisomal membrane protein PEX14 and an FITC-labeled goat antirabbit secondary antibody (Chemicon). Cells were visualized using a Nikon Eclipse E800 fluorescence microscope equipped with an FITC filter. Images were captured with a Photometrics Coolscan CCD camera (Roper Scientific) and processed using V++ Precision Digital Imaging software (Digital Optics).

**Fuzzy c-means algorithm**

The fuzzy c-means (FCM) algorithm (Bezdek, 1981) seeks to partition a dataset \( \{x_1, x_2, \ldots, x_m\} \), where \( x_m = (x_{m1}, x_{m2}, \ldots, x_{mk}) \), \( m = 1, 2, \ldots, M \), into a specified number of fuzzy regions which are represented by the corresponding cluster centers. The degrees of each \( x_m \) that belong to different clusters are characterized by the corresponding fuzzy membership grades taking real values between 0 and 1.

In principle, the FCM maximizes the following objective function:

\[
J(U, c_1, \ldots, c_N) = \sum_{y=1}^{N} \sum_{m=1}^{M} \mu_{ym}^{\alpha} d_{ym}^2,
\]

(1)

where \( M \) is the number of data points, \( N \) is the number of clusters, \( U \) is the \( N \times M \) fuzzy membership matrix, \( \mu_{ym} \in [0, 1] \) is the fuzzy membership grade that indicates the degree \( x_m \) belongs to the fuzzy region \( y \), \( d_{ym} \) is a distance measure between cluster center \( c_y \) and data point \( x_m \), and \( \alpha \in [1, \infty) \) is the fuzzy exponential weight.

The computations of the cluster centers and the partition matrix \( U \) are updated by an iterative procedure which is described as follows:

1. Given the degree of fuzziness \( \alpha \) and initial membership matrix \( U \) with random values of \( \mu_{ym} \in [0, 1] \) subjected to

\[
\sum_{y=1}^{N} \mu_{ym} = 1, \quad \forall m = 1, \ldots, M.
\]

2. Update initial cluster centers

\[
c_{y}^{j+1} = \frac{\sum_{m=1}^{M} \mu_{ym}^{\alpha} x_m}{\sum_{m=1}^{M} \mu_{ym}^{\alpha}}.
\]

(2)

3. Update fuzzy membership functions

\[
\mu_{ym} = \frac{1}{\sum_{z=1}^{N} \left( \frac{d_{ym}}{d_{zm}} \right)^{2/(\alpha-1)}}.
\]

(3)

where, using the \( L_2 \) norm, \( d_{ym} \) is given by

\[
d_{ym} = ||x_m - c_y||_2.
\]

(4) Compute the objective function according to Equation (1). If it converges or its improvement over the previous iteration is below a certain threshold then stop the iterative process. Otherwise, go to step 2.

**Estimating the number of clusters**

Taking a first look at the image (412 x 357) as shown in Figure 1, there appear to be two classes to be segmented. These two classes are the background pixels and the peroxisome puncta. If we apply the well-known Otsu’s thresholding method (Otsu, 1979) and the FCM, with the number of clusters \( N = 2 \), to segment the gray image of Figure 1, we obtain Figures 2 and 3 which are the results given by Otsu method and the FCM, respectively. It can be seen that both results overestimate the spot sizes and highlight noise and outliers. These are due to the low contrast of the image and particularly the fluorescence around the peroxisome spots. We therefore need to add another cluster to represent the fluorescent-shadow...
pixels, i.e. the number of classes will now be three instead of two. As Otsu method only works for gray-scale images with two classes, we now apply the FCM with \( N = 3 \) and obtain another result as shown in Figure 4. This result shows some improvement over that obtained by the FCM with \( N = 2 \). However, overestimation of spot areas and touching spots still remain to some extent. We will tackle these problems by a strategy for sharpening the fuzziness of the peroxisome cluster, an aspect-ratio criterion and quadtree decomposition, which are presented in the following subsections.

**Focusing image spots by sharpening fuzzy regions**

Based on the concept of a fuzzy set (Zadeh, 1965) and the notion of the Shannon’s entropy (Shannon and Weaver, 1948), the measure of fuzziness of a fuzzy set was initially defined by DeLuca and Termini (1972) as follows:

1. The fuzziness of \( A = 0 \) if \( A \) is a crisp set, i.e. \( \mu_A(x) \in \{0, 1\}, \forall x \in X \).

2. The fuzziness of \( A \) is maximum when \( \mu_A(x) = 0.5, \forall x \in X \).

3. The fuzziness of \( A \) is greater than or equal to that of \( A^* \) if \( A^* \) is a sharpened version of \( A \), i.e. \( \mu_{A^*}(x) \geq \mu_A(x) \) if \( \mu_A(x) \geq 0.5 \); and \( \mu_{A^*}(x) \leq \mu_A(x) \) if \( \mu_A(x) \leq 0.5 \).

Let \( \mu_p(x) \) be the fuzzy membership grade that indicates how a possible pixel \( x \) belongs to the set containing all the peroxisome images, we then apply the notion of the measure of fuzziness to sharpen the fuzzy region of interest (peroxisome) by defining

\[
\mu^*_p(x) = \begin{cases} 1 & \mu_p(x) \geq \delta_\mu, \\ 0 & \mu_p(x) < \delta_\mu, \end{cases}
\]

where \( 0.5 < \delta_\mu < 1 \) is a fuzzy membership threshold.

What we discuss next is how to get an appropriate value for \( \delta_\mu \) in order to obtain good sharpened peroxisome spots which can make the task of isolating touching spots easier. To fix a concrete idea, let \( \mu_{c^*}(x) \) be the fuzzy membership grade of a pixel \( x \) belonging to the peroxisome cluster \( c^* \). We can say that an optimal value of \( c^* \) must be some value between the least, denoted by \( f_{\min}(x|c^*) \) and the most, denoted by \( f_{\max}(x|c^*) \), bright intensities which are to be assigned to \( c^* \). Of course, it is difficult to determine \( f_{\min}(x|c^*) \) readily; however, \( f_{\max}(x|c^*) \) is immediately available, i.e. by checking the membership grade of the brightest pixel of the whole image assigned to \( c^* \) given by the FCM. We therefore select \( \delta = \mu_{c^*}(x^*) \), where \( f(x^*) \) is the maximum intensity value, because \( \mu_{c^*}(x^*) \) represents the brightest and the least bright pixels which are to be assigned to \( c^* \). Finally, each segmented peroxisome region will be filled up in case there are any holes in the region.

Figure 5 shows an improved sharpened version, in comparison with the result as shown in Figure 4. By applying the sharpening procedure defined in Equation (4)—the segmented spot areas are sharpened and brought closer to the real spot areas than the former segmented results; in addition, more outliers are removed in this sharpened version.

**Isolating touching spots by aspect-ratio criterion**

We define an aspect ratio of a spot image \( p \), based on which touching spots can be isolated, as

\[
r(p) = \frac{w_{\min}(p)}{w_{\max}(p)},
\]

where \( w_{\min}(p) \) and \( w_{\max}(p) \) are the minimum and maximum widths of the spot area and \( w_{\min}(p) \geq \) the maximum width of the estimated smallest spot size.
Fig. 5. Segmentation of image A by sharpening FCM with three clusters.

The procedure for splitting touching spots is described as follows.

(1) Given a spot image $p^i$, $i = 1, \ldots, I$, where $I$ is the number of segmented spots which are greater than an estimated smallest spot image.

(2) If $r(p^i) < 0.5$, then split $p^i$ into two subimages $p^i_1$ and $p^i_2$ at the location of $w_{\text{min}}(p^i)$.
   (a) If $p^i_g$, $g = 1, 2$, is greater than an estimated smallest spot size and $r(p^i_g) < 0.5$, then separate $p^i_g$ into two subimages $p^i_{g,1}$ and $p^i_{g,2}$ at the location of $w_{\text{min}}(p^i_g)$.
   (b) Repeat step (a) for all subimages $p^i_{g,1,2}$ where each subscript takes the values from 1 to 2.

(3) Repeat steps 1 and 2 for all $p^i$.

Adaptive segmentation by quadtree partition

What has been described above regarding the fuzzy membership threshold $\delta_{\text{seg}}$ expressed in Equation (4) is a nonadaptive case for the FCM-based segmentation because $\delta_{\text{seg}}$ remains the same for the whole image. We notice that, first, the regions of interest (peroxisome) occupy only part of the image; second, if we apply the FCM to segment these images with a large size of 1392 x 1040 pixels, the computational time will be considerably long and not so effective for real applications; and third, as an important factor regarding the parameter $\delta_{\text{seg}}$ whose sensitivity depends on the brightest pixel and if the brightest pixel is not chosen locally, then many real spots having relatively low intensities will be subjected to false rejection. We therefore apply the scheme of quadtree partition that has been largely used for fractal image compression (Fisher, 1994), to iteratively divide the whole image into quadrants so that both segmentation quality and speed will be much enhanced, particularly the second and third issues. By doing this, the segmentation now becomes an adaptive process in which the threshold $\delta_{\text{seg}}$ will be estimated differently for each image quadrant.

The image will be partitioned into quadrants (upper left, upper right, lower left and lower right) if its variance is equal or greater than a splitting threshold $\delta_{\text{var}}$, that is

$$\text{var} = \frac{1}{N} \sum_{n=1}^{N} [f(x, y) - \bar{f}(x, y)]^2 \geq \delta_{\text{var}}, \quad (5)$$

where $N$ is the total number of pixels within a (sub)image, $f(x, y)$ and $\bar{f}(x, y)$ are the pixel intensity and the average pixel intensities of the (sub)image, respectively.

In order to avoid carrying out the FCM-based segmentation of subregions containing all background pixels, we define another decision parameter, denoted as $\delta_{\text{seg}}$, based on which the FCM-based segmentation will be performed if the maximum intensity value within a subimage is greater than a threshold, i.e. the decision is to do the fuzzy segmentation if

$$f_{\text{max}}(x, y) \geq \delta_{\text{seg}}, \quad (6)$$

where $f_{\text{max}}(x, y)$ is the maximum intensity value within a particular subimage respectively, and $\delta_{\text{seg}}$ can be experimentally estimated.

Procedure for extracting peroxisome spots

(1) Convert the given RGB image into intensity image $I$.

(2) Use the quadtree technique to partition $I$ into a set of $Q$ subimages: $I = I_1 \cup I_2 \cup \cdots \cup I_Q$.

(3) Do FCM-based segmentation for each $I_k$, $k = 1, \ldots, K$, where $K$ is the number of quadtree-split images which contain peroxisome spots(s), i.e. $K \leq Q$.

(4) Sharpen and fill up spot areas (if there are any holes).

(5) Isolate touching spots in each $I_q$, $q = 1, \ldots, Q$, using the aspect-ratio criterion.

(6) Assemble all segmented versions of $I_q$, $q = 1, \ldots, Q$ to obtain the whole segmented version of $I$.

RESULTS AND DISCUSSION

In addition to the illustrations, which have been presented in the foregoing sections, showing some advantages of our FCM-based segmentation approach, we further test our proposed method and compare the results with other methods for image spot extraction. For the current FCM analysis, we select $\alpha = 2$ and $\delta_{\text{seg}}$ to be the round off of $(255/2)$ for all cases, for extracting peroxisome image spots on several real images. The reason for choosing the value of $\alpha = 2$ is based on the most popular choice for the FCM analysis found in literature as there is no certain analytical ground for selecting the right value of this parameter at present (Bezdek, 1981; Chi et al., 1996) and for $\delta_{\text{seg}}$, being half of 255 is based on pre-experiment on a few images from which $\delta_{\text{seg}}$ was found
fairly constant and only large discrepancy on the values of this parameter will turn on or turn off the decision for the FCM analysis.

Figure 6 shows the intensity version of an RGB color image (412 × 357) that contains fluorescent-stained peroxisome spots. Edges of these spots are fuzzy due to low contrast, also some of the spots are connected to each other. Some fluorescent stains may misrepresent spots (false spots) for simple segmentation methods. Figures 7–10 show the segmented versions using Otsu thresholding method, FCM with three clusters, ImageJ that is a public-domain image processing software and can be downloaded from the web (http://rsb.info.nih.gov/ij/) and our proposed FCM-based segmentation method. It can be seen from these figures that the results obtained from both Otsu’s thresholding, straightforward FCM and ImageJ that uses a thresholding method developed by Ridler and Calvard (1978), show false as well
as overestimated peroxosome spots; whereas our proposed method yields the segmentation results that are quite close to the actual spots and can also isolate touching spots.

As another experiment, Figure 11 shows the original image where the task of spot extraction is more difficult than the earlier case, in that the image contains many noisy spots. Figures 12–15 show the segmented versions using Otsu’s thresholding method, FCM with three clusters, ImageJ and the proposed FCM-based segmentation. The result obtained by our approach is more accurate than the other three methods. Few small fading peroxosome spots are omitted by our method whereas relatively large number of false spots are detected by the other three methods, particularly by Otsu’s thresholding and the ImageJ.

Figures 16–17 shows the Canny edges (Canny, 1986) of the results obtained from ImageJ (Figure 14) and the proposed method (Figure 15), respectively. Again it can be seen that the edges of the peroxosome spots obtained by our method are much more realistic than those of the ImageJ. Spot areas obtained from the ImageJ are significantly overestimated from the actual spot sizes shown in Figure 11; whereas the proposed FCM-based segmentation approach yields a more accurate result with the spot areas being close to the actual spots. Touching spots are also isolated by the proposed FCM-based method.

Figures 18–20 show the full-size (1392 × 1040) versions of the original image, ImageJ-based and the proposed FCM-based segmentation results respectively. Not only is the proposed method able to yield more accurate spot areas, but also able to suppress noise and isolate touching spots.
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Fig. 16. Canny-edge image of segmentation by ImageJ (iterative thresholding).

Fig. 17. Canny-edge image of segmentation by proposed FCM-based method.

Fig. 18. Original image D.

Fig. 19. Segmentation of image D by ImageJ (iterative thresholding).

Fig. 20. Segmentation of image D by proposed FCM-based method.

Fig. 21. Original image E.

Figures 21–23 show the full-size (1392 × 1040) versions of another original image, ImageJ-based, and the proposed FCM-based segmentation results, respectively. Conclusion for this case are the same as stated above for the results shown in Figures 18–20.

It is mentioned that from all of the above presented results, the extraction of the number of spots and the spot sizes obtained by our method gained the most favor of several biologists at the Eskitis Institute for Cell and Molecular Therapies and the School of Biomolecular and Biomedical Sciences.
Griffith University. From various results, the method is reasonably robust against noise as many low-contrast puncta were detected, isolated and their sizes were more accurately estimated than the other methods.

CONCLUSIONS

We have presented an effective algorithm for extracting fluorescent peroxisome puncta in fuzzy images where the contrast is low, spots are touching and background is mixed with fluorescence, which make standard techniques for image segmentation or edge detection ineffective. We have tested our proposed FCM-based algorithm with real image data and obtained favorable results and in all cases have superior results in comparison with existing methods. This algorithm is expected to prove useful for the analysis of different cell compartments following fluorescence microscopy.

REFERENCES