Published in final edited form as:

J Neurosci Methods. 2013 February 15; 213(1): 84–98. doi:10.1016/j.jneumeth.2012.12.011.

Automated condition-invariable neurite segmentation and synapse classification using textural analysis-based machine-learning algorithms

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Abstract

High-resolution live-cell imaging studies of neuronal structure and function are characterized by large variability in image acquisition conditions due to background and sample variations as well as low signal-to-noise ratio. The lack of automated image analysis tools that can be generalized for varying image acquisition conditions represents one of the main challenges in the field of biomedical image analysis. Specifically, segmentation of the axonal/dendritic arborizations in brightfield or fluorescence imaging studies is extremely labor-intensive and still performed mostly manually. Here we describe a fully automated machine-learning approach based on textural analysis algorithms for segmenting neuronal arborizations in high-resolution brightfield images of live cultured neurons. We compare performance of our algorithm to manual segmentation and show that it combines 90% accuracy, with similarly high levels of specificity and sensitivity. Moreover, the algorithm maintains high performance levels under a wide range of image acquisition conditions indicating that it is largely condition-invariable. We further describe an application of this algorithm to fully automated synapse localization and classification in fluorescence imaging studies based on synaptic activity. Textural analysis-based machine-learning approach thus offers a high performance condition-invariable tool for automated neurite segmentation.

Keywords

neurite; synapse; imaging; segmentation; machine learning; textural analysis

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INTRODUCTION

Recent advances in high-resolution live-cell imaging techniques together with the development of a new generation of fluorescent indicators have dramatically enhanced researchers' ability to study fundamental questions in neuronal and synaptic function. Viral trans-synaptic tracers, for example, can be used to reveal precise local neuronal circuit connectivity (Callaway, 2008), while the development of the Brainbow mice with nearly a hundred color variances of neuronal labels may allow complete reconstructions of large-scale circuit architecture (Livet et al., 2007). Moreover, introduction of fluorescent probes that report synaptic activity such as FM1-43 or synapto-pHluorins, has allowed access to fundamental questions in synaptic physiology including neurotransmitter release, synaptic vesicle recycling and synaptic plasticity at the level of individual synaptic boutons and even individual synaptic vesicles (Cochilla et al., 1999; Peng et al., 2011; Ryan, 2001; Yuste et al., 2000).

One of the main challenges in the further development and application of these advanced imaging approaches has been the lack of automated, generalizable and condition-invariable image analysis tools for neuronal tracing/segmentation and synapse localization (Ascoli, 2008; Donohue and Ascoli, 2010; Helmstaedter et al., 2008; Meijering, 2010). Segmentation and reconstruction of a 3D dendritic arbor of a single neuron, for example, takes days and often weeks for a skilled human operator. Even 2D experiments in neuronal cell culture typically include identification of several hundred synaptic boutons in each frame in time-lapse sequences that often contain several thousand frames, bringing the total amount of data points to tens of thousands per experiment. Such prohibitively large data sets emphasize the need for automated analysis of imaging data with little or no human intervention. As emphasized by the Neuroinformatics Grand Challenge (Ascoli, 2008), however, even in the presence of possible experimenter's bias and the exhaustive nature of the work, manual neurite segmentation and selection of synaptic region of interests (ROI) is generally preferred because of the failure of existing image processing techniques under imperfect image acquisition conditions (e.g., inhomogeneous contrast, limited resolution, and background fluctuations). In addition, high-resolution imaging studies in which synapse localization is required are characterized by large intrinsic variability in synapse size and often a low signal-to-noise ratio, leading to a potentially significant bias in selection of a correct subset of functionally important synaptic ROIs to be analyzed.

While a number of semi-automated and fully automated neuronal segmentation algorithms have been developed and successfully used in several applications, their performance typically depends on image acquisition conditions (Ascoli, 2008; Donohue and Ascoli, 2010; Meijering, 2010). Intensity-gradient-based segmentation algorithms, for example, are often considered the efficient way to segment large volume of digital microscopy images. Consequently, several adaptive-thresholding-based techniques that use either raw image intensities, image gradients or filtered responses have been proposed for segmenting brightfield and fluorescent microscopy images (Broser et al., 2008; Janoos et al., 2009; Narro et al., 2007; Pool et al., 2008). However, such techniques are quite sensitive to global and local maxima, noise level, type of fluorescent probes and specimen conditions. As a result it is often difficult to obtain consistent, clean segmentation of neurite regions from digital microscopy images using thresholding-based techniques that employ intensity values (Figure 1) (Ascoli, 2008; Meijering, 2010). The goal of the current study thus was to develop a framework for a fully automated neuronal segmentation that can differentiate neurites from the background independently of the image acquisition conditions.

Many of the existing limitations in image analysis tools, and particularly dependence on image acquisition conditions, could be addressed by statistical machine-learning techniques.

In this work, we describe a textural-analysis-based machine-learning framework for automated neurite segmentation using an example of brightfield images of cultured neurons. In the following sections we provide a detailed description of how we adapt machine-learning techniques to approach the problem of neurite segmentation. We describe how we use the training data to learn the textural information pertaining to neurite- and non-neurite regions and subsequently use the learned information to segment any given image. We measure the performance of the proposed framework in comparison to an expert's manual segmentation and demonstrate that this approach combines a high accuracy with high sensitivity and specificity. Moreover, using a wide range of image acquisition conditions we show that this textural analysis-based approach is largely condition invariable. Finally, we demonstrate the use of this approach for activity-based localization/classification of synapses that permits separation of synaptic vs. axonal/dendritic regions. We believe that this framework for segmentation and localization can be easily extended to other forms of neuroanatomical microscopy with appropriate changes in training data.

METHODS

Neuronal cell culture

Dense primary cultures of hippocampal neurons were created as previously described (Deng et al., 2011; Kaech and Banker, 2006; Peng et al., 2011). Briefly, hippocampi were surgically isolated from the P0-P1 pups, dentate gyrus was removed, and tissue was dissociated following a brief papain treatment. Neurons were plated on astrocyte feeder layers at ~50-75K/ml in the Neurobasal media supplemented with B27 and held in culture for 3 weeks.

Imaging

2-3 weeks old cultured hippocampal neurons were mounted on an inverted Olympus microscope (IX70, Olympus, Japan) and images were acquired with 60X, 1.4NA oil immersion objective. For fluorescence imaging, FM1-43 was excited with a Xenon lamp via a filter cube containing 480/40 nm excitation filter, 515nm dichroic mirror and 575/60nm emission filter. Fluorescence or brightfield signals were captured using a cooled EM CCD Hamamatsu camera and Simple PCI software (Hamamatsu Corp., PA, USA). Neurons were stimulated using a pair of electrodes positioned ~1cm apart in the bath and controlled by the computer software via Master-8 stimulus generator (A.M.P.I, Israel). All recordings were performed at 37°C using a whole-microscope incubator equipped with a rapid feedback temperature controller (In Vivo Scientific, St Louis, MO).

Immunofluorescence detection of axons, dendrites and synaptic components

Axons and dendrites were stained for tau and MAP2, respectively; synaptic contacts were detected with synaptophysin (pre-synaptic) and PSD95 (post-synaptic) staining. Hippocampal cultured neurons were fixed at DIV14-18 with 20% sucrose in 4% phosphate buffered paraformaldehyde solution. Fixed neurons were incubated with primary antibodies for 30 minutes, washed three times with PBS, incubated with alexa-labelled secondary antibodies, mounted with Prolong mounting medium (Invitrogen) and imaged at 60x. Antibodies used were as follow: anti-tau (Millipore, 1:50), anti-MAP2 (Millipore, 1:2000), anti-synaptophysin (Synaptic Systems, 1:500), anti-PSD95 (Synaptic Systems, 1:100), Alexa-labelled secondary antibodies (Invitrogen, 1:1000).

RESULTS

1. Automatic Threshold Segmentation

To evaluate performance of segmentation algorithms and to identify the important unresolved problems in automated neurite segmentation, we examined a simple case of brightfield images of live hippocampal cell cultures grown at high density on glass coverslips for 2-3 weeks (Figure 1A). These cultures offer a useful experimental system to test segmentation approaches, since they exhibit several problematic features such as large variability of background due to properties of underlying astrocyte feeder layer, and a wide range of apparent neurite sizes due to the presence of axonal/dendritic bundles common in high-density mature neuronal cultures (Figure S1).

To provide a performance reference, throughout the paper we compared our texture-based segmentation approach to several existing automatic thresholding algorithms. Segmentation based on automatic thresholding is a commonly used approach to discriminate objects from background. Many such thresholding algorithms have been developed for different conditions (Huang and Wang, 1995; Li and Lee, 1993; Otsu, 1979; Ridler and Calvard, 1978). Here we used the auto threshold plugin to ImageJ which uses 16 different algorithms. It is important to note that for any particular image we evaluated performance of all 16 thresholding algorithms, but for comparison with our approach we used only the one that produced the best result for that image, and thus there is no single thresholding algorithm that can produce the reported results for all images. In the case of the neuronal culture's brightfield images, simple application of automatic threshold methods results in suboptimal segmentation (Figure 1B). The performance of these algorithms can be significantly improved, however, by applying two preprocessing techniques, rescaling and filtering. Since neurites have a variable position relative to the focal plane throughout the image, they appear both darker and lighter than the background. We therefore segmented an image which results from the absolute difference from the median of the original image (Figure 1C). Additionally Gaussian blur filter was applied to reduce false segmentation of noise (Figure 1C). The results of auto threshold of such preprocessed images showed a significantly improved performance (Figures 1D,F and S2). However, for the purposes of neurite segmentation, we noted that non-homogenous background (Figure 1D) and inability to adequately adjust to variability in neurite size (Figure 1F, note loss of majority of fine neurites) hampered automatic threshold performance (Table 1, and see below for performance metrics).

2. Discriminative Modeling

To achieve optimal segmentation without user intervention we combined two powerful pattern recognition techniques, namely a discriminative model-based machine-learning approach with adaptive filter selection scheme and a set of morphological post-processing filters. Previous studies have shown that discriminative model-based 3D and 2D-textons could be effectively used to classify textural surfaces with high level of accuracy even under variable acquisition conditions (Kandaswamy et al., 2011). In this work we adapt such discriminative model-based machine-learning technique to our problem of segmenting neurite arborizations under variable live-cell imaging conditions. Our goal was to use this machine-learning tool to first quantitatively learn the characteristics of neurite regions on the image surface and the inherent and extraneous variations of the neurite representation under different conditions, and then to use this information for automatic identification and segmentation of neurite regions in any given image. For the quantitative learning process, hereafter referred as Learning Stage, we employed a collection of training images that were manually segmented and verified by imaging experts. In the following subsections, we provide the description and the formulation of our Learning and Segmentation stages.

2. 1. Filter Selection For Neurite Segmentation In Brightfield Images

In natural images, the problem of pattern recognition under non-ideal conditions such as changes in viewing direction, deformations, and changes in ambient illumination is often solved by obtaining invariant descriptors of the image, such as, for instance, illumination- or rotation-invariant features (Kandaswamy et al., 2011; Leung and Malik, 2001; Varma and Zisserman, 2005). From a machine-learning point of view, however, the same problem is often solved by obtaining, or learning, optimal descriptors that capture the variations within individual textural classes (intra-classes), while still maintaining sufficient inter-class distances. In our case, this is equivalent to finding optimal texture descriptors that capture the intra-class variations (such as variations within neurite regions and variations within different background regions) while establishing significant differences between background (non-neurite regions) and neurite regions.

Previous studies that examined the effects of surface variations (such as those due to shadows, illumination/viewing conditions and occlusions in 3D textured surfaces) indicated that careful selection of appropriate filter sets (Adjeroh et al., 2007b; Varma and Zisserman, 2005) is essential for constructing the basic functions of a discriminative modeling paradigm that can effectively minimize intra-class variations while maintaining sufficient inter-class distances. While typical filter sets required as many as 38-48 filters (Kandaswamy et al., 2011; Leung and Malik, 2001; Varma and Zisserman, 2005), selection of an optimal set of filters based on the features of interest was shown to effectively reduce the required number of filters to 15, while achieving better segmentation performance than larger filter sets (Adjeroh et al., 2007). In this selection process, a smaller set of filters was chosen based on filters' correlations. Small sub-images were randomly selected from the training images. These sub-images were filtered using the complete filter set and the correlations of filter values in each image were calculated. Filters for which the average correlation between filter values over the sub-image set was larger than 0.9 were grouped together. From each group, filters with a maximal response were selected for the smaller filter set used for analysis.

In this work we adapted a similar correlation-based filter selection technique and derived twelve optimal sets of filters out of 124 filters constructed as variations (of the σ and W parameters) of three filter groups, namely the Gabor filter group

$$g_d(x, y) = \frac{1}{2\pi\sigma_x\sigma_y} \exp \left[- \left(\frac{x^2}{2\sigma_x^2} + \frac{y^2}{2\sigma_y^2} \right) + j2\pi(W_x x + W_y y) \right]$$

where W is the modulation frequency; Edge filter group

$$g_d(x, y) = \frac{1}{2\pi\sigma_x\sigma_y} \exp \left[- \left(\frac{x^2}{2\sigma_x^2} + \frac{y^2}{2\sigma_y^2} \right) + \exp(xy) \right]$$

LoG filter group

$$g_d(x, y) = - \frac{1}{\pi\sigma_x^2\sigma_y^2} \left[1 - \left(\frac{x^2}{2\sigma_x^2} + \frac{y^2}{2\sigma_y^2} \right) \right] \exp \left\{ - \left[\frac{x^2}{2\sigma_x^2} + \frac{y^2}{2\sigma_y^2} \right] \right\}$$

The optimization of filter selection we used in the current study offers a two-fold advantage in being both computationally effective while also providing optimal segmentation performance as we describe in detail below.

2.2. Learning Stage

We started our formulation by defining the brightfield image as a single channel grayscale image I of size $N \times N$. We constructed our discriminative modeling paradigm using a collection of filters g_d that were selected using a correlation-based filter-pruning technique as described above (and see Adjero et al., 2007). Filter responses of a given training image I and filters g_d can be defined as time-domain convolution between the image and the filter:

$$F_d = \sum_k \sum_l I(x, y) g_d^*(x - k, y - l) \quad d = [1, 2, \dots, P]$$

where P is the number of filters. The computed filtered responses were stacked to form a feature space $\widehat{F}_s$ of size $P \times N^2$, where each pixel position (x, y) was described using a vector corresponding to different filtered responses:

$$\widehat{F}_s^i(x, y) = \begin{bmatrix} F_1^i(x, y) \\ F_2^i(x, y) \\ F_3^i(x, y) \\ \vdots \\ F_P^i(x, y) \end{bmatrix}$$

where $\widehat{F}_s^i(x, y)$ represents the stacked feature vector from position (x, y) of the i^{th} training image. In order to make sure that neither the outliers in pixel values nor small anomalous regions in the images skew the cluster centers, we removed such pixel values and their corresponding vectors $\widehat{F}_s$ from the feature space. We used the pixel sorting method, similar to the one used in gray-scale normalization algorithm, for finding the pixel values that are considered outliers (occurred less than 1%). The feature space $\widehat{F}_s$ from a given training image was then separated into neurite regions and non-neurite regions and then clustered using *k-means* clustering algorithm to obtain D number of cluster centers from the neurite regions and B number of cluster centers from background regions, as illustrated in Figure 2. Since segmentation quality converges to a limit as the number of clusters and computational time increase (Figure S3), the smallest number of clusters ($B + D = 200$) that produced the optimal segmentation quality was chosen to optimize computational efficiency (Figure S3). The clustering operation was then repeated for T_N number of training images. The computed cluster centers (hereafter referred as textons) from T_N training images can be written as:

$$\vec{\alpha} = \vec{\alpha}_{S, b \mid b \rightarrow 1-(D+B)}^i = \left[\vec{\alpha}_{S,1}^i, \vec{\alpha}_{S,2}^i, \vec{\alpha}_{S,3}^i, \dots, \vec{\alpha}_{S,D}^i, \vec{\alpha}_{S,D+1}^i, \vec{\alpha}_{S,D+2}^i, \dots, \vec{\alpha}_{S,D+B}^i \right]$$

where $\vec{\alpha}_{S,b}^i$ is a vector of length P with index b ranging between 1 to $(D + B)$ from the i^{th} training image. Extracted textons from each training image were stored and used accordingly for segmenting any given image.

2.3. Segmentation Stage

To segment a new image, the image was filtered using the same set of filters (g_d) and all the filtered responses were stacked to form a three-dimensional image. Each pixel position (x, y) of the new three-dimensional image was represented as a vector $\widehat{F}_q^i(x, y)$, where the vector elements were the corresponding filtered responses of that position. Based on the Euclidean distance between the vector $\widehat{F}_q^i(x, y)$ and the textons $\widehat{\alpha}_{q,b}^i$, the pixel position (x, y) was assigned an index b . Thus the texton-mapped version of the input image can be written as:

$$M^i(x, y) = \underset{b}{\operatorname{argmin}} \left[\frac{1}{P} \sum_{q=1}^P \left(\widehat{F}_q^i(x, y) - \widehat{\alpha}_{q,b}^i \right)^2 \right] \quad b = [1, 2, \dots, D, D+1, D+2, \dots, D+B]$$

The resultant Euclidean distances corresponding to each training image and mapping were stored as:

$$E^i(x, y) = \min \left[\frac{1}{P} \sum_{q=1}^P \left(\widehat{F}_q^i(x, y) - \widehat{\alpha}_{q,b}^i \right)^2 \right] \quad b = [1, 2, \dots, D, D+1, D+2, \dots, D+B]$$

Final aggregate mapping of the microscopy image constructed from the entire set of training images can be described by:

$$\mathbb{E}(x, y) = \min_i \left[E^i(x, y) \right]$$

$$\mathbb{M}(x, y) = M^i(x, y) \mid \underset{i}{\operatorname{argmin}} \left[E^i(x, y) \right] \quad i = [1, 2, \dots, T_N]$$

This stage of processing that combined multiple versions of segmentation from different training sets provided a significant improvement in segmentation accuracy (see Quality Measures and Segmentation Accuracy below, and Figure 3A,C). The final segmented image was computed in the form of a binary image by assigning 1's to the regions of brightfield image that were assigned neurite textons and 0's to the regions that were assigned non-neurite textons:

$$I_{Seg}(x, y) = \begin{cases} 1 & \mid \mathbb{M}(x, y) \leq D \\ 0 & \mid \mathbb{M}(x, y) > D \end{cases}$$

Hereafter, image surface that has been assigned 1's will be referred as $\mathbb{D}$ and the non-neuritis region will be referred as $\mathbb{B}$.

3. Quality Measures And Segmentation Accuracy

Performance of the proposed segmentation framework was evaluated using a diverse set of quality measures to provide an objective and multifaceted evaluation. All performance measures were computed against manual segmentation, which was verified for accuracy by imaging experts. First, we evaluated our machine-learning-based segmentation algorithm from the point of robustness and measured the importance of selecting an optimal set of

filters tuned specifically for our neurite segmentation problem. In this analysis we used Receiver Operating Curves (False rejection rate vs. False acceptance rate) (Duda et al., 2001) to measure how effectively the regions of interest, the neurites, were separated from the background (Figure 3A). This analysis showed that segmentation performance improved significantly by using an optimal subset of filters selected based on the feature of interest (using correlation based filter selection technique) relative to the previously used, larger but less specific filter sets. Similarly, a post-processing stage combining multiple versions of segmentation from different training sets further improved segmentation accuracy (Figure 3A). This is also evident from the distributions of neurite- and background- assigned textons (Figure 3B), which show clear separation with small overlap. We performed a detailed analysis of the dependence of segmentation quality and its variability on the number of training images used (Figure 3C). This study was performed with 12 training images in which the segmentation quality and its variation were measured based on all possible combinations of n images from the overall training images. Segmentation quality as measured by the adjusted rand index (Hubert and Arabie, 1985) (and see below) showed an increase and saturation as the number of training images increases. In addition, the variability in performance, estimated by the standard deviation of the adjusted rand index, decreases with the number of training images, indicating that relying on a small training set leads to inconsistent segmentation quality. Both trends demonstrate the positive impact of combining textons from multiple training images on segmentation quality.

Second, we compared the results of our segmentation framework with other popular segmentation algorithms (available via ImageJ software). For this analysis we used a traditional set of quality measures, namely specificity, sensitivity and accuracy as well as Rand index (Rand, 1971) and adjusted Rand index (Hubert and Arabie, 1985) measurements of clustering similarity. Specificity was measured as the ratio of the number of True Negatives and sum of True Negatives and False Positives, where True Negatives are the number of pixel positions from non-neurite regions of brightfield images that are correctly identified by the algorithm as non-neurite, and False Positives are the number of pixel positions corresponding to non-neurite regions that are falsely identified by the algorithm as neurite regions. Sensitivity was measured as the ratio of number of True Positives and the sum of True Positives and False Negatives. True Positives are the number of pixel positions that are manually segmented to be neurites and identified by the algorithm as neurite and False Negatives are the pixel positions that are manually segmented to be neurites and identified by the algorithm as non-neurites. Accuracy was measured as a ratio between total number of correctly classified pixel positions (sum of True Negatives and True Positives) and the total number of pixels positions. Rand index (Rand, 1971) measures the similarity between two clustering methods (tested and manual) through evaluation of paired cluster association. Adjusted Rand index (Hubert and Arabie, 1985) compensates for random contribution to Rand index by subtracting the expected value of random clustering, such that in case of random clustering the adjusted Rand index converges to 0. The two latter measures are similarity indices commonly used to compare partitions (segmentation) of a data set. It was recently shown that following correction of contributions due to chance (such as in adjusted rand index), different similarity indices provide an equivalent quality measure (Albatineh et al, 2006). We note that all of the quality measurements we used here rely on single or pairwise pixel comparisons and do not account for structural properties such as continuity, volume, neurite number etc. However, such structural measures are very numerous and specific for the desired application. Therefore no single structural measure is general enough for the assessment of segmentation quality in our case.

The performance of our segmentation algorithm in comparison with 5 other proposed approaches is summarized in Table 1. The proposed machine-learning-based approach had stronger performance than thresholding techniques by achieving 90% accuracy while

reaching the highest levels of specificity (87%) and similar levels of sensitivity (93%). Moreover, the Rand index and adjusted Rand index were also much higher for our segmentation algorithm compared to other approaches (Table 1). Figure 4 shows the visual comparison between manual segmentation and our machine-learning-based segmentation approach for two examples of brightfield images of neuronal cell culture acquired under different experimental conditions and segmented with ~90% accuracy. As evident in these examples, the largest contribution to segmentation errors came from the false acceptance of cell debris, which had similar textural characteristics as that of normal cellular processes. Further improvement in accuracy could be achieved in future studies by adding an analysis module which is trained to recognize the debris characteristics by considering their distinct morphological features and using symmetry considerations. We note that although a small percentage (~2%) of neurites had discontinuities in the original images (Figure 5C), presumably due to going out of focus, accurate segmentation did not require the detected neurites to be continuous.

Taken together, the above analysis demonstrates that the proposed approach provides a major improvement in accuracy of neurite segmentation in brightfield images of live cells without sacrificing sensitivity and specificity. We also note that we examined applicability of our approach to segmenting brightfield images of fixed cells, which are characterized by poor contrast. We found a reasonable level of success with $\sim 78.4 \pm 1.6\%$ accuracy, which also represents a significant improvement over the performance of the thresholding algorithms (data not shown). This result suggests that our approach may also be useful in studies combining live-cell imaging with immunofluorescence or other forms of imaging in fixed cells.

4. Repair of Neurite Discontinuities

One limitation common to many segmentation algorithms, including the one presented here, is the presence of discontinuities in segmented neurites (Ascoli, 2008; Donohue and Ascoli, 2010; Helmstaedter et al., 2008; Meijering, 2010). Such discontinuities limit application of our and other segmentation algorithms to automatic neurite reconstruction. We thus developed an additional post processing stage to repair the observed discontinuities. This post processing addressed two types of discontinuities, short- and long-range ones and used a different approach in each case (Figure 5A,B). Short discontinuities were solved by consecutive application of morphological operations dilation followed by erosion of the image. This approach efficiently sealed small gaps with sizes of 4 pixels or less (Figure 5B). Repair of long discontinuities was based on two considerations: (i) discontinuities appeared predominantly in the thin neurites and (ii) small sections of these thin neurites between points of intersection appeared nearly linear under high magnification conditions of our images. The linearity property of the neurites allowed us to use the Hough transform (Duda and Hart, 1972) for long-range repair (Figure 5A). This transform represents each line in the image by its angle and distance from the origin. In transformed space each line (angle and distance) is represented by the total number of neurite pixels on that line. The local maxima in transform space represent lines which were segmented mostly. Along each of these lines width of segmentation was calculated and discontinuities were identified as 0 width. This analysis was performed in a sliding window to avoid preferred detection of large neurites. To prevent erroneous repairs, several properties of the neurites were measured: mean width, variance of width (consistency) and asymmetry of the segmented neurite with respect to the detected line. These parameters were needed to ensure that only neurites that are parallel to the line are filled, while neurites that cross the detected line are not considered.

To quantify the improvements in reducing neurite discontinuities by this post-processing repair, we examined 617 neurite segments in 14 segmented images before and after the

repair. Original texton-based segmentation without repair resulted in ~87% of branches having no discontinuities. In addition, ~2% of discontinuities in neurites were “true discontinuities” in the original brightfield images, predominately due to neurites going out of focus (Figure 5C). Our post processing was able to repair ~70% of discontinuities (Figure 5A,B,D). With this post processing, our segmentation approach thus had a ~95% overall success rate in segmenting neurites without discontinuities.

5. Condition Invariance

One of the major existing limitations of automated segmentation tools is strong dependence on image acquisition conditions (Ascoli, 2008; Meijering, 2010). To test to what extent the machine-learning texton-based segmentation we proposed depends on image acquisition conditions, we analyzed two sets of images acquired either at a wide range of different exposure times or a wide range of camera gain settings (Figure 6). Whether exposure or gain were varied, these different acquisition conditions span the entire visible range from very dark to very bright images (Figure 6A-C). Every image in the set was segmented and compared to manual segmentation performed on a single image of the set taken under optimal conditions. The texton-based algorithm performance was not strongly affected by the changes of imaging conditions, for both varying exposure time (Figure 6D) or camera gain (Figure 6E). The same image sets were also segmented using an automatic threshold algorithm. The threshold performance was much more dependent on acquisition conditions with strong decrease in quality for the brightest images (Figure 6D,E). Most importantly, the texton-based segmentation provided an improved segmentation throughout the entire range of image acquisition conditions. We obtained the same results of near condition invariable performance of texton-based algorithm in analyses of 3 additional sets of images with varied gain or exposure. These results suggest that within the parameter set of imaging conditions tested, texton-based neurite segmentation is largely condition invariable.

6. Application of Automated Segmentation for Synapse Classification

The challenge of segmenting neurite regions is, in many cases, only a first step within the more complex problem of extracting useful information from digital images in brightfield or fluorescence microscopy studies. In other words, an automation process such as the framework proposed here can often become part of a more complex application in which neurite segmentation is used as a basis to define other ROIs and perform further image analyses. For example, several recent studies have revolutionized our views on synaptic transmission by showing that neighboring synapses share a large common pool of synaptic vesicles that travel along the axons and can be presumably used by various synapses upon increased synaptic activity (Staras et al, 2010, Darcy et al, 2006). Due to inability of current image analysis tools to separate synaptic regions from the axonal regions in fluorescent or brightfield images, such studies can only be currently performed with sufficient accuracy using an electron microscopy approach in fixed tissue. The dynamic properties of such a global vesicle pool and its use during synaptic activity remains unknown, and could only be studied using live imaging techniques if a precise separation of synaptic regions from neurite regions can be achieved. Here we demonstrate an application of our segmentation algorithm as an initial step to building a fully automated activity-based synapse classifier by combining neurite segmentation in brightfield images with existing synapse detection algorithms in fluorescent images to precisely localize and distinguish synaptic and extra-synaptic locations based on synaptic activity.

To label active synapses, cultured hippocampal neurons, after 14-18 days in culture, were first loaded with lipophilic fluorescent indicator FM1-43 via compensatory endocytosis using a single round of 10 Hz, 200 pulse stimulation, then washed, and unloaded with

another round of strong stimulation (1000 pulses at 10 Hz). The location of active synapses was determined by comparing two images, one taken following a loading step immediately before the second stimulus (Stained image), and the second image taken following a round of destaining stimulation (Destained image). This protocol is widely used to specifically identify the synapses that uptake and release the dye. The second round of destaining is necessary to reduce the artifacts from non-specific dye loading. Brightfield images were also taken at the beginning and the end of each experiment for subsequent neurite segmentation.

To identify active synapse locations we adopted a previously developed fluorescent puncta detection algorithm U-track (Jaqaman et al., 2008) that has been shown to effectively work for localizing puncta in the appropriate size range. Detection in U-track is performed under the assumption that fluorescence image results from the sum of multiple point sources and therefore the shape of the objects in the image should be Gaussian with fixed standard deviation determined by the properties of the microscope. Puncta are selected from the local maxima in the image by properties of the hessian. Each of these puncta is fitted using Gaussian mixture model fit to possibly detect multiple particles in each puncta (Jaqaman et al., 2008). Since each synapse with multiple labeled vesicles is not a single point source, we used multiple rounds of peak detection with progressively increasing width of Gaussian fits to locate point of interests (POIs) of multiple sizes varying from 100nm to 1000nm, representing typical range of synapse sizes in hippocampal neurons (Schikorski and Stevens, 1997). A pair-wise proximity measure was then used to cluster together the POIs that were within $0.5\mu\text{m}$ from each other and therefore represented multiple detections of the same synaptic terminal (Figure 7). In this approach, each correctly located POI had following features associated with it:

$P^V(x, y)$ – Size of a Gaussian fitted in the chosen point

$P^A(x, y)$ – Area of POI centered at (x, y) measured in number of pixels

$P^P(x, y)$ – Peak intensity detected in the region

These features were then used to form a vector,

$\vec{P}^F(x, y)^I$ – Feature vector (histogram of pixel values) computed from a window of size

$N_S \times N_S$ at location (x, y) from image I , where N_S is the maximal synapse size in pixels.

We estimated the synapse detection accuracy of this approach at ~97%, as compared with manual detection by an imaging expert. We note that this is slightly above the accuracy of our segmentation, suggesting that synapses may have a higher density on more robust thicker neurites that have a tendency to be segmented more accurately.

Given that synaptic activity can be characterized as a function of change in FM1-43 intensity upon stimulation over time, we computed synaptic activity at any given POI as a function of change in the distribution of the pixel values around that POI. Figure 8I,J shows the difference between feature vectors extracted from Stained and De-Stained neurite regions and Stained and De-stained background regions. We then used the mutual information based dissimilarity measure to quantify this difference in features and thus estimate the level of synaptic activity. The dissimilarity measure can be described as:

$$D_{MI}(x, y) = -P^{\vec{F}}(x, y)^S \log \left(P^{\vec{F}}(x, y)^D \right)$$

where $P^{\vec{F}}(x, y)^S$ and $P^{\vec{F}}(x, y)^D$ are the POIs at location (x,y) in Stained and Destained images. In order to account for dye bleaching we computed a similar dissimilarity measure, D_{Bleach} , between Stained and De-stained images, using the distribution of pixels from non-neurite regions (as determined by the segmentation stage). Any POIs with D_{MI} greater than that due to bleaching observed in non-neurite regions (D_{Bleach}) was considered to contain significant neuronal activity. Thus, the accuracy of the segmentation algorithm plays an important role in determining not just the neurite regions but also in subsequently analyzing fluorescent images. This global dissimilarity measure was then used to classify fluorescently labeled puncta in FM1-43 stained neuronal cell culture into three groups based on the level of synaptic activity and puncta localization (Figure 9) as (i) those that were not significantly active, but localized to neurites; (ii) those with average synaptic activity, and (iii) those with above-average synaptic activity (both groups (ii) and (iii) were also localized to neurites). The first group may represent the packets of fluorescently labeled synaptic vesicles in the “super pool” that were traveling from one synapse to another (Staras et al, 2010, Darcy et al, 2006) and therefore did not undergo activity-dependent release (de-staining). The groups (ii) and (iii) presumably represent synaptic terminal locations that underwent activity-dependent vesicle release to varying degrees. The observed variable levels of dye release correspond well to known variability in release properties in these neurons (Fernandez-Alfonso and Ryan, 2004; Peng et al., 2011; Sara et al., 2005; Schikorski and Stevens, 1997). This approach thus allows the precise separation of synaptic and extrasynaptic locations based on a combination of neurite segmentation and synaptic activity patterns and may thus permit future studies of vesicle transport and sharing among synapses in live neurons. In addition to the example of synapse classification presented here, we suggest that automated texton-based neurite segmentation can be combined with many other image analysis tools in various applications where precise localization of neuronal structural features is of interest.

DISCUSSION

Here we described an automated machine-learning approach based on textural image analysis that allows accurate segmentation of neurite arborization patterns in 2D brightfield images. This approach provides a significant improvement in both accuracy and specificity over existing thresholding-based segmentation algorithms. Importantly, the texton-based algorithm maintains a high level of performance under a wide range of image acquisition conditions indicating that it is largely condition invariable. Using an example of synapse localization and classification, we show that our approach can be effectively combined with secondary image analysis algorithms to address many more complex questions in neuronal imaging studies. Since our approach is based on machine learning, we suggest that it can be easily adapted to many other types of neuroanatomical and functional microscopy, given the appropriate changes in the training dataset.

Texton-based machine-learning approach for neurite segmentation

Machine-learning algorithms have been successfully used for pattern recognition and information retrieval purposes both in natural image processing (Dana et al., 1999; Leung and Malik, 2001; Varma and Zisserman, 2005) and in biometric applications such as face recognition and iris segmentation under various viewing conditions (Kandaswamy et al., 2007a), and even under extreme non-ideal image acquisition conditions (Jurie and Triggs, 2005). Recently such techniques in combination with textural analysis algorithms have also been successfully applied to the field of automated biomedical image analysis (Adjero et al., 2007a; Narasimha et al., 2009). Yet, so far such approaches have had very limited application to the field of neuroinformatics and neuronal segmentation (Helmstaedter et al., 2008; Meijering, 2010). Here we applied a texton-based machine-learning approach to perform neurite segmentation in brightfield images of live cultured hippocampal neurons.

Cultured neurons represent a simple yet useful model system that provide several features, including background variations and a wide range of neurite widths, that are common in more complex systems and imaging environments. These properties of a simplified model system highlighted the common difficulties that existing automated segmentation approaches experience in the segmentation task (Figure 1). Throughout the paper we compared our texton-based segmentation to several existing automatic threshold algorithms and found that background variations and variability of neurite widths indeed strongly hampered the performance of available threshold based approaches (Figure 1). This is qualitatively reflected in the low specificity and high sensitivity of these algorithms. Inhomogeneous background causes problems to any algorithm based on pixel value, since such algorithms have no means of distinguishing high background reading from neurite. This problem is however easily addressed by our textural analysis algorithm, since background inhomogeneity is typically less directed than the neurites, and thus distinction is simple. Thin neurites also pose a problem for thresholding methods since they tend to be less contrasted from the background than the thicker neurites. Hypothetically, this could be partially rescued by performing local thresholding in regions where only thin neurites exist. However, since the thin and thick neurites are intrinsically intermixed, local thresholding would not provide a major improvement in reality, and also has a drawback of a greater false detection in empty regions. Our analysis further suggests that spatial properties of the neurites, such as orientation, connectivity and branching did not contribute strongly to segmentation failures by thresholding algorithms, as expected from the single-pixel nature of this family of methods. Spatial properties could, in principle, affect performance of texture-based approaches, but we did not observe increased errors or breaks at the branching points or at any specific orientation (data not shown).

A major advantage of using a texton-based machine-learning technique for pattern recognition applications, such as recognizing the regions of neurites, is that textural units for a particular region of interest can be “objectively” learned from a collection of training inputs from an imaging expert. In addition, this approach of deriving a texton-model for a particular type of textural surface can also be extended to learn variations of the textural surface under different acquisition conditions by effectively controlling the variability in the training data. In particular, texton-based algorithms that use textural pattern information surrounding each pixel position to identify and classify textures have been shown to have more robust performance in natural images compared to the techniques that use simple pixel/intensity values or its gradients (Kandaswamy et al., 2011). In application to neurite segmentation, we found that texton-based approach offers a significant improvement in specificity relative to the existing algorithms, reaching over 90% (Table 1). Most importantly, unlike the existing approaches which commonly offer either high specificity or high sensitivity but not both, texton-based approach combines high specificity with high sensitivity as well as a superior accuracy (Table 1). Its much improved performance relative to many existing approaches is also evident by using more generalizable metrics of segmentation quality known as rand index and adjusted rand index (Table 1). The texton-based machine learning algorithm thus represents an accurate tool for neurite segmentation with high specificity and sensitivity, as we demonstrate on the example of brightfield images of cultured neurons. While applications of this approach to other types of neuronal imaging is beyond the scope of the current study, the major improvements in several segmentation quality measures we assessed, suggest that this approach may be useful in many other types of neuroatomical applications.

Repair of Discontinuities

An important limitation of many segmentation algorithms including threshold or edge detected segmentations, as well as the texton-based approach presented here, is the presence

of discontinuities/breaks in segmented neurites (Donohue and Ascoli, 2010). This problem is particularly critical to neural reconstruction algorithms that depend on extensive post processing. Several approaches for repair discontinuities have been developed that often depend on the specific applications and types of imaging data (Narro et al., 2007; Xiong et al., 2006). Many skeletonization algorithms include removal of side branches and connection of disconnected segments as one of post-processing operations (Vallotton et al., 2007). Our texton-based segmentation approach successfully segments ~87% of neurites without discontinuities. With an additional post processing tool that we developed to address both short- and long-range breaks (Figure 5), our approach reached a ~95% rate of segmenting neurites without discontinuities. This result illustrates that even rather simple additional post-processing can repair a significant proportion of neurite discontinuities. We recognize that this additional post-processing repair approach is somewhat specific to our application and may not be applicable to every existing type of images. In this case, our segmentation can be combined with the existing repair tools (Narro et al., 2007; Xiong et al., 2006).

Condition invariability

Another major limitation of automated segmentation tools is dependence on image acquisition conditions (Helmstaedter et al., 2008; Meijering, 2010). Our analysis suggest that unlike threshold-based approaches, the texton-based segmentation maintained high levels of accuracy and specificity across a wide range of conditions with little variation in performance (Figure 6). Thus texton-based approach offers a major advantage over existing algorithms in being nearly condition invariable. Such robust performance could offer advantages in many types of neuronal and biomedical imaging applications characterized by variable or non-ideal imaging conditions such as, for example, dynamics studies with imaging at high frame rates (Fernandez-Alfonso and Ryan, 2004; Klingauf et al., 1998; Sara et al., 2005), reduced signal-to-noise ratio intrinsic to single-particle and single synapse studies (Murthy et al., 1997; Peng et al., 2011), time-lapse fluorescence studies with extensive variations in image intensity due to phlorophore bleaching (Cochilla et al., 1999; Ryan, 2001), or in cases of significant background variations common to many neuronal imaging problems in cell cultures and *in vivo* (Belichenko and Dahlström, 1995; Bertling et al., 2012; Cochilla et al., 1999; Ryan, 2001; Yuste et al., 2000).

Applications to neuronal reconstruction and other image processing problems

Automated segmentation of neurites is often a first step in many automated neuronal reconstruction algorithms (Ascoli et al., 2007; Donohue and Ascoli, 2010). Many of the existing approaches rely on threshold or edge detection in their initial segmentation (Cuntz et al., 2008; Selinummi et al., 2006). The initial segmented images are then further processed using techniques such as skeletonization, watershed etc. to generate a morphological object (Ascoli, 2008; Cannon et al., 1998; Helmstaedter et al., 2008; Meijering, 2010). Application of texton-based segmentation as an initial step in such cases could provide a significant improvement of initial segmentation and therefore allow a more robust and reliable reconstruction at the following stages.

In addition, our approach may also serve as a first stage in more complex microscopy studies in which neurite segmentation is used as a basis to define other ROIs and perform further image analyses. Indeed, we demonstrate one application of our approach to localization and classification of synapses in fluorescence images by their activity levels, a task that maybe of interest to a wide range of researchers interested in synaptic function. Indeed, numerous high-resolution studies of synaptic transmission are performed in cultured neurons and critically depends on correct localization of active synapse ROIs (Fernandez-Alfonso and Ryan, 2004; Peng et al., 2011; Sara et al., 2005). Given the presence of several

hundred puncta per field of view in a typical neuronal cell culture image labeled for synaptic activity (Fernandez-Alfonso and Ryan, 2004; Klingauf et al., 1998; Sara et al., 2005), accurate and automated separation of synaptic ROIs from debris in culture is an essential and formidable task that texton-based segmentation can be used to address. Moreover, the dynamic properties of many physiological processes inside synaptic boutons and surrounding axonal areas are highly divergent, such as, for example, the kinetics of cytoskeletal transport (Cingolani and Goda, 2008; Peng et al., 2011). A precise separation of axonal and synaptic regions based on initial texton-based segmentation as we described here, can provide a significant improvement in quality of data analysis in such studies. Furthermore, many recent imaging studies of synaptic function and plasticity focused on processes localized to dendritic spines (Bertling et al., 2012; Bhatt et al., 2009), which are believed to serve as a separate biochemical compartment. Accurate automated segmentation of dendritic and spine morphology should be possible using a texton-based segmentation approach with an appropriate training dataset, and may thus provide a major improvement in image analysis of dendritic processes. In summary, we suggest that machine-learning texton-based neurite segmentation framework we proposed here can be combined with many other image analysis tools in various applications where precise localization of neuronal structural features is of interest.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by grants to V.K. from the Esther A. and Joseph Klingenstein Fund, Edward Mallinckrodt Jr. Foundation, Whitehall Foundation and in part by National Institutes of Health Grant RO1NS060709 to V.C. We thank Diana Owyong and members of the Klyachko lab for the constructive comments on the manuscript. We also thank Drs. Danuser and Jaqaman for kindly sharing their U-track software.

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Highlights

- Automated machine-learning approach allows accurate segmentation of neurite arborizations.
- Proposed approach provides a significant improvement in accuracy and specificity.
- The algorithm is largely condition invariable.
- Application for automated synapse classification in fluorescence images is presented.

Figure 1. Need for advanced techniques for automated brightfield image segmentation

A. Brightfield image of neuronal cell culture. Scale bar shown is the same for all images.

B. Automatic threshold segmentation of the image shown in (A). The “Otsu” algorithm available as a plugin in Image J was used.

C, D. Automatic threshold performance (**D**) was significantly improved by two preprocessing stages of rescaling and filtering (**C**). However, background variations strongly impacted the algorithm performance as significant background areas were erroneously segmented as neurites.

E, F. Another example of automatic threshold segmentation of an image (**E**) after preprocessing. This example highlights another problem of automated thresholding in that many thin neurites are missed in the segmentation.

Figure 2. Schematic representation of the discriminative-modeling-based machine-learning algorithm used for automated neurite segmentation

A. The block diagram for the Learning Stage showing how the generic descriptors corresponding to different regions (neurite and background) of brightfield images are learned in the form of cluster centers (textons). This part of the algorithm is a one-time process and performed only once for every single training image.

B. In the Segmentation Stage, the extracted textons are stored and used for segmenting any given brightfield image.

Figure 3. Receiver Operating Curves (ROC) of segmentation algorithms

A. The ROCs (False rejection Rate vs. False Acceptance Rate) of different segmentation algorithms. The optimal set of filters selected for our particular type of problem shows improved performance in comparison to the previously proposed set of filters. Adding a layer of additional processing that combines multiple versions of segmentation from different training sets further improves the performance of the segmentation algorithm by decreasing the Equal Error Rate (EER) to 10.14% from 18.26%.

B. The distribution of texton-index from texton-mapped images after the additional processing stage. The separation between neurites and background support high pixel segmentation quality. **C.** Analysis of segmentation quality as a function of the number of training images. The average adjusted rand index (black) increases and saturates with the increase in number of training images, while standard deviation (blue) of the adjusted rand index decreases.

Figure 4. Neurite segmentation in brightfield images acquired under different imaging conditions

A,B. *Left:* Two examples of brightfield images acquired with different contrast levels. Scale bar shown is the same for images. *Middle:* Manual segmentation of the two images by an imaging expert. *Right:* The segmentation results of the machine-learning texton-based approach. A training set of 4 images (cut in quarters into 16 smaller sub-images actually used for training) was chosen randomly from a larger set of acquired images. Proposed approach offers high specificity, while maintaining high levels of sensitivity and accuracy (Table 1).

Figure 5. Post-processing stage for repair of discontinuities

A. Original image (left), texton-based segmentation (middle) and texton-based segmentation with a post-processing stage for repair of discontinuities (right). Both short- and long-range repaired discontinuities are shown in red. Five long-range repaired discontinuities are highlighted by arrows.

B. Same as (A) for another sample image highlighting repair of short-range discontinuities. Several short-range discontinuities are evidenced in original texton-based segmentation (middle), and their repair is highlighted (right).

C. Example of several “true” discontinuities that occur in the original images, predominately due to neurites going out of focus.

D. Percentage of neurite segments segmented without discontinuities using texton-based segmentation approach with and without the post-processing discontinuities repair tool. 617 neurite branches from 14 segmented images were evaluated. Post-processing stage repaired ~70% of discontinuities, resulting in overall ~95% success rate.

Figure 6. Performance of texton-based machine-learning algorithm under different image acquisition conditions

A-C. Sample images of the same neuronal culture acquired under three different exposures (left) resulting in low light, underexposed image (**A**), optimal image (**B**) and overexposed image (**C**). Texton-based segmentation of the corresponding images (middle), and automatic threshold segmentation of the corresponding images (right). Texton-based segmentations include a post-processing stage for repair of discontinuities, shown in red. Scale bar shown is the same for all images.

D. Performance evaluation of segmentation results under different acquisition conditions, as assessed by accuracy (left), modified Rand index (middle) and specificity (right) calculated for segmentations of images acquired under a wide range of exposure times. The post-processing repair of discontinuities shown in (**A-C**) was not taken into account in this evaluation.

E. Same as **(D)** for a wide range of camera gain settings.

Automated synapses localization and activity based synapses classification

Figure 7. The schematic representation of synapse localization and classification algorithm First, all possible synaptic regions (POIs) in FM1-43 stained images were identified using fluorescent particle detection algorithms as described previously (28). Multiple rounds of peak detection for puncta of variable size in the range of 100-1000nm were performed. Second, identified POIs were tested for proximity using Euclidean distance measure of the Cartesian coordinates and clustered together if the POIs were within 0.5 μ m. For each POI, corresponding feature vector (histogram of pixel values) were extracted from both Stained and De-stained images. Only POIs within the regions segmented as neurites were considered further and classified into three different groups based on the level activity observed (see Figure 7).

Figure 8. Mutual information based dissimilarity measure for synapse classification

A-D. The FM1-43 stained, de-stained, brightfield and manually segmented images of a sample neuronal cell culture image. Note that to make sure we can detect significant difference in distribution between pixel values from stained and de-stained regions and to make sure that this difference is not due to imperfections of automated segmentation, in this analysis we used an example of manual segmentation by an imaging expert. Scale bar is 2 μm and is the same for all images.

E, F. Change in pixel values pertaining only to the background regions.

G,H. Change in pixel values pertaining only to neurite regions.

I. Distribution of pixel values pertaining only to the background regions. As expected due to the effect of fluorescent dye bleaching, we saw a small increase (0.06 to 0.08) in the number of pixels with lower values in the De-stained image compared to that of Stained image.

J. The distribution of pixel values in neurite regions from stained (red) and de-stained (black) images. The observed shift in the distribution is mainly due to synaptic activity and the corresponding destaining process. The mutual information between the two distributions is used to quantify the difference between destaining caused by bleaching and destaining caused by synaptic activity.

Figure 9. Activity-based synapse classification

A. An example of automatically segmented image with localized POIs. Scale bar is 3 μm and is the same for both images.

B. Activity-labeled regions are classified into three color-coded groups based on their location and the level of synaptic activity. Puncta marked red have a low or insignificant level of synaptic activity (less than or equal to bleaching), but are localized to neurites. Puncta marked green show a significant level of synaptic activity. Puncta marked blue show above-average activity. The latter two groups likely represent synaptic terminals, while the former may correspond to vesicle packages traveling in axons.

Table 1

Comparison of popular segmentation algorithms with the proposed algorithm: Tabulation of the quality measures for five different segmentation algorithms (in terms of Rand index, adjusted Rand index, specificity, sensitivity and accuracy) are provided for comparison. ImageJ software plug-in modules were used to obtain the alternative segmentations

Technique	Rand Index	Adjusted Rand Index	Accuracy	Sensitivity	Specificity
Isodata thresholding	0.57	0.14	0.69	0.98	0.37
Fuzzy thresholding	0.70	0.41	0.82	0.95	0.66
Minimum cross entropy thresholding	0.68	0.36	0.80	0.96	0.59
Mean thresholding	0.71	0.41	0.82	0.95	0.67
Cluster thresholding	0.60	0.20	0.72	0.99	0.40
Proposed algorithm	0.83	0.65	0.90	0.93	0.87