Short communication

A novel algorithm for optimal image thresholding of biological data

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Article info

Article history:
Received 30 June 2010
Received in revised form 26 August 2010
Accepted 26 August 2010

Keywords:
Image processing
In vivo microscopy
2-Photon imaging
Thresholding
Laser-scanning Confocal

Abstract

With the proliferation of both in vivo and in vitro microscopy techniques in the neurosciences, increased attention has been placed on the development of image analysis techniques. As experiments can produce large numbers of high bit depth images, automated processing methods have become necessary for handling these data sets. Thresholding, whereby a high bit depth image is converted into a binary image in order to identify a feature of interest, is one such standard automated technique; but the method of selecting an appropriate threshold value is far from standard. We present a novel algorithm, maximum correlation thresholding (MCT), that thresholds images accurately and efficiently without relying on any assumptions of the statistics of the image. As MCT produces thresholded images that preserve the most salient elements in the image, the algorithm performs as well as a trained user on a range of neurobiological data and in a variety of noisy conditions or when preprocessing steps preceded the thresholding operation. Our method will thus allow neuroscientists to automate image thresholding using a robust, computationally efficient algorithm, ultimately aiding in accurate image quantification and analysis.

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1. Introduction

Modern imaging techniques in neuroscience have allowed unprecedented access to the structure and function of cells in the mammalian nervous system. With the proliferation of in vitro and in vivo microscopy techniques, large data sets of high bit depth neuronal images are frequently generated. In each image for example, processes such as the dendrite, or the dendritic spines, must be identified and tracked to study a biological phenomenon of interest. Generally, these experiments involve multiple imaging sessions of the same dendritic volume over multiple days, increasing the number of images that need to be processed and analyzed. As such, these large data sets necessitate automated image processing techniques for analysis, identification and characterization of anatomical structures. Consequently, whether the goal is to identify structural changes in vivo (Gruetzendler et al., 2002; Mizrahi et al., 2004; Trachtenberg et al., 2002), as we have described, or characterize physiological events in vitro and in vivo (Kapoor and Urban, 2006; Ohki et al., 2005), a first step is usually image thresholding. Thresholding is the process whereby fluorescent intensities above a value are considered signal and those below that value are considered noise. Choosing the correct threshold value is therefore critical; values that are too low can result in an overestimation of features of the signal, and values too high can exclude faint low intensity features that are essential for understanding the neuronal process being studied. A number of algorithms exist to implement a strategy for choosing an optimal threshold. Classically, the approach employed by most of these algorithms is to calculate an optimal threshold value by examining the statistics of the image histogram, e.g. (Otsu, 1979; Ridler, 1978) a calculation that implicitly relies on a segregation of peaks (one peak for background, one peak for signal) within the histogram to arrive at a threshold value. For some types of anatomical in vivo 2-photon data, such separable peaks may not be easily identifiable as the features are generally sparse, faint, and exist with low signal-to-noise ratios.

To deal with these specific image processing challenges, and more broadly, to develop a way of selecting an ideal threshold, we present a novel thresholding algorithm, maximum correlation thresholding (MCT). First, our algorithm provides an optimal solution forbinarizing images independent of any a priori assumptions about the statistics of the image. Second, we validated our method by comparing the performance of MCT with a trained user familiar with thresholding neuronal data over a wide array of images with various statistical features, acquired using qualitatively and
2. Materials and methods

2.1. MCT algorithm

We define an image $X$ as a vector $x$ where $x_n$ corresponds to the $n$th pixel:

$$x = x_1, x_2, x_3, \ldots, x_n$$

We define another vector $y$ where $y_n$ corresponds to the $n$th pixel:

$$y = y_1, y_2, y_3, \ldots, y_n$$

such that $y_n = \begin{cases} 1 & \text{if } x_n > \bar{x} \\ 0 & \text{else } x_n \leq \bar{x} \end{cases}$, where $i$ is a threshold value. From this the correlation coefficient is given as:

$$corr(x, y) = \frac{\sum_{i<j} n_i (j - \bar{x}) + (N_i/N_m) \sum_{i>j} n_j (j - \bar{x})}{\sqrt{\left(\sum_{i<j} n_i (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)\left(\sum_{i>j} n_i (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)}}$$

(1)

$$corr(x, y) = \frac{\sum_{i<m} n_i (j - \bar{x}) + (N_i/N_m) \sum_{i>m} n_j (j - \bar{x})}{\sqrt{\left(\sum_{i<m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)\left(\sum_{i>m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)}}$$

(2)

(3)

with $x$ as the original image and the thresholded image $y$ at the threshold value $(i - 1)$, $n_i$ as the number of pixels with the value $j$ and $\bar{x}$ as the mean pixel value of the image. In addition we define $N_m$ as the total number of pixels in the image and $N_i$ as the number of pixels above the threshold value $(i - 1)$.

We rewrite our correlation where $x$ is the original image and $y(i)$ is the image thresholded at a value of $i$:

$$corr(x, y(i)) = \frac{\sum_{i=m}^{m} n_i (j - \bar{x}) - (N_i/N_m) \sum_{i=m}^{m} n_j (j - \bar{x})}{\sqrt{\left(\sum_{i=m}^{m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)\left(\sum_{i=m}^{m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)}}$$

(4)

(5)

with $-N_i/N_m \sum_{j=1}^{m} n_j (j - \bar{x})$ going to 0 and that subsequently reduces to the final implementation form:

$$corr(x, y(i)) = \frac{\sum_{i=1}^{m} n_i (j - \bar{x})}{\sqrt{\left(\sum_{i=1}^{m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)\left(\sum_{i=1}^{m} (j - \bar{x})^2 + n_j ((N_i^2/N_m) + (1 - (2/N_m)N_j/(N_m - N_j)))\right)}}$$

(6)

From this, a vector $t = corr(x, y(1)), corr(x, y(2)), corr(x, y(3))$ \ldots corr($x, y(m)$) is defined for all pixel values between 1 and $m$ and the optimal threshold value is given as the $t_m$ that maximizes $corr(x, y(m))$. We note that the algorithm described is applicable to images of all bit depths.

2.2. Computational utility

Though previous approaches have suggested using cross-correlations to select threshold values (Brink, 1989), we have arrived at a computationally superior method for calculating the optimal threshold and tested it on both normal and noisy image data. Because our approach operates on a modified equation of the correlation coefficient (Eq. (3)), it is independent of the image size (which had a significant effect on the time taken to calculate correlation). Let $P$ represent the number of pixels in the image $M$ the number of grayscale values of the image. Let theta $\theta$ be a function for the runtime of the algorithm. Then, the correlation coefficient between the original image and the thresholded image requires $\theta(PM)$ calculations. Our algorithm reduces the calculation to $\theta(P) + \theta(M)$ which improves the speed of the thresholding problem significantly.

2.3. In vivo 2-photon imaging

In vivo 2-photon images were acquired through a modified Zeiss LSM510 Meta NLO microscope (Zeiss, Germany) coupled to a Mira 900 Ultra-Fast TiS laser system pumped by a Verdi V10 diode laser (Coherent, Santa Clara, CA). All images were acquired with an excitation wavelength of 890 nm.

2.4. Animal procedures

All animal experiments were carried out in accordance with approved protocols for care and use of animals at the Carnegie Mellon University.

For dendritic processes, GFP-M and GFP-O mouse neocortex (Feng et al., 2000) was imaged by methods similar to those previously described (Trachtenberg et al., 2002). Prior to surgery and imaging, animals were anesthetized with IP injections of ketamine and xylazine and maintained on equal volume oxygen and nitrous oxide supplemented with isofluorane. Heart rate was monitored throughout the procedure, and animals were given 1 cc of 5% dextrose Ringers subcutaneously during the procedure. A region overlying either the primary visual cortex or the somatosensory cortex was exposed by removal of overlying skull while preserving the dura. Following this, a 0.5–1.5% agarose solution was placed over the exposed brain and held down with a glass cover slip and affixed to the skull. Dental cement was then used to construct an imaging chamber.

In vivo images of developing thalamo-cortical axons were acquired from the neonatal ferret. Briefly, exposure of dorsal thalamus via a small lesion through the overlying somatosensory cortex and hippocampus (Crowley and Katz, 1999, 2000) was followed by pulse injection of a lentivirus expressing GFP either downstream of the EF1-α (Tranzyme, NC) or CAMKII promoters (provided by Dr. Pavel Osten) into the LGN (Osten et al., 2007).

Following sufficient expression time (between 1 and 3 weeks), an agarose chamber was placed over primary visual cortex and two photon images were acquired as described above.

For LGN images, retinal ganglion cell axons were labeled with eye injections of Cholera Toxin β-subunit conjugated to Alexa 488, 555, or 594 (Invitrogen, CA). Following transport of the label for 24–48 h, animals were sacrificed and 50 μm horizontal sections were made of the lateral geniculate nucleus as previously described (Huberman et al., 2002, 2003). 12 bit images were acquired on a Zeiss Axiosplan2 (Zeiss, Germany) with a Hamamatsu ORCA 2 ER-AG CCD camera (Hamamatsu Photonics, Japan) using IPLab (Scanalytics, MD).

For calcium imaging, methods were adapted for use in ferrets based on previously described work in mice (Kapoor and Urban, 2006). Briefly, ferrets between P20–30 were anesthetized with IP injections of ketamine and xylazine and sacrificed before 300 μm coronal sections of primary visual cortex were made. Sections were incubated in 50 μl of oxygenated Ringers for 30 min with 3 μl of 0.01% pluronic (Invitrogen, CA) and 5 μl of fura-2 AM (Invitrogen, CA). After loading, slices were transferred to a chamber and spontaneous and evoked activity was imaged using custom software written in Igor Pro (Wavemetrics).

2.5. Image analysis

Images were cropped for analysis using Photoshop (Adobe, CA) and all analysis was performed using MATLAB (Mathworks, MA). Included in the supplementary materials is the MATLAB code for MCT (MCT.m). The Otsu algorithm was implemented using the built-in functionality of MATLAB (the function graythresh was called to determine a threshold value and im2bw was used to threshold the image. We used the MATLAB V7.5 version to implement...
the thresholding) and the Ridler-Calvard algorithm was implemented using code provided by Dr. Robert Murphy and confirmed with code downloaded from the MATLAB central file exchange (the file isodata.m was implemented by Dharmesh Ramachandram and acquired from the MATLAB central file exchange URL = http://www.mathworks.com/matlabcentral/fileexchange/loadFile.do?objectId=3195&ObjectType=file). Statistics on analyzed images were performed using Microsoft Excel (Microsoft, WA) or MATLAB.

3. Results

MCT iterates through all integer values of a histogram's dynamic range, thresholds the histogram at each of these values, and calculates a normalized correlation coefficient value (Eq. (5)) between the thresholded histogram and the original image histogram. Our algorithm selects the threshold value that maximizes the correlation coefficient between the two images over the image bit depth range.

In order to test the MCT algorithm, we collected two photon in vivo images of dendritic spines from GFP-M and GFP-O mice (Feng et al., 2000) and in vivo images of thalamo-cortical axons labeled with a lentivirus construct expressing eGFP in primary visual cortex of the developing ferret (Fig. 1A). In addition, to confirm that our algorithm was applicable to a wide array of neurobiological data types, we also collected fixed tissue wide-field fluorescence images of retinal ganglion cell axon projections in the lateral geniculate nucleus (LGN) labeled with Alexa fluorophores (Fig. 2A2) and slice images of cells labeled with the calcium indicator Fura 2 (Fig. 2A3).

In vivo images of a thalamo-cortical axon from a ferret (Fig. 1A1) and an apical dendrite of a mouse (Fig. 1A2) and the two representative curves of the correlation coefficients between the original image (image Fig. 1A1 corresponds to curve Fig. 1B1), (image Fig. 1A2 corresponds to curve Fig. 1B2) and the thresholded image are shown (Fig. 1B1 and B2). The threshold value at the peak (black arrow, Fig. 1B1 and B2), corresponding to the maximum normalized correlation coefficient, is then applied to the original image to produce a binary thresholded image (Fig. 1C1 and C2). Our approach does not depend on any a priori assumptions of the image's statistics, such as the separability of two peaks in the pixel histogram distribution (Fig. 1D1 and D2), and consistently produced images wherein fine structures including axons and dendrites (red arrows) were preserved as in the original (Fig. 1C).

In order to confirm that our approach was not an artifact of implementation, but instead exploited a feature of the distribution of the data, we reasoned that there must be some relationship between the normalized correlation curve (Fig. 1B1 and B2) and the pixel histogram (Fig. 1D1 and D2) that produces such optimally thresholded images. First, to scale the distribution of pixel values, we plotted the natural log of the pixel histogram distribution (Fig. 1E1 and E2) and then compared these curves with the derivative of the correlation coefficient curve (Fig. 1F1 and F2). The shape of the derivative curve of normalized correlation coefficients recapitulated the shape of the pixel histogram distribution, and the crossing of the derivative curve from the positive to negative (black arrow, Fig. 1F1 and F2) identified the peak of the correlation coefficient curve (black arrow, Fig. 1B1 and B2).

As thresholding is generally used to identify biological features as a prerequisite for subsequent analysis (Torborg and Feller, 2004), we wished to validate our technique by comparing the MCT algorithm to manually thresholded images where a specific feature of interest was selected for by a trained user. From a collection of over 1000 images acquired of in vivo and in vitro data, we selected a random assortment and manually thresholded those images for comparison to MCT. We grouped the image types into three classes of data; in vivo images of neurites (e.g. Fig. 2A1), images of retino-geniculate projections labeled with Alexa fluorophores terminating in the lateral geniculate nucleus (LGN) of the thalamus (e.g. Fig. 2A2), and images of cells from a coronal slice of the visual cortex of the ferret loaded with the Ca^2+ indicator Fura-2 (e.g. Fig. 2A3). The classifications reflected both qualitatively (because of the methods of acquisition and the spatial and temporal resolution differences between image classes) and quantitatively different data types (revealed in the different histogram distributions). These distinctions allowed us to determine the extent to which the MCT algorithm could threshold various types of data. Within each class (in vivo axons and dendrites N = 10, LGN N = 5, Ca^2+ N = 5) we manually thresholded the images (Fig. 2B1–B3) and compared the manually identified features with those thresholded through our MCT algorithm (Fig. 2C1–C3) both in appearance (Fig. 2D1–D3) and correlation (Fig. 2E1–E3 and F). When we overlaid manually
thresholded images (pseudo-colored green) of dendrites (Fig. 2D1) or fluorescently labeled axons in the LGN (Fig. 2D2) with images produced by the MCT algorithm (pseudo-colored red) we found that there was a remarkable agreement between the two (yellow pixels). Notably, features selected by the user that were labeled by the fluorescent indicator were the same features that the MCT selected.

We found that manually thresholded images were not significantly different to those using the MCT algorithm in images of axons and dendrites (Fig. 2E1, \( P = 0.702, N = 10 \), ANOVA) by measuring the correlation coefficient. This was done to quantify the similarity of the MCT image and the manual image. Furthermore, we did not observe any significant differences in the LGN images (Fig. 2E2, \( P = 0.383, N = 5 \), ANOVA). Finally, though we observed no significant difference in the correlation coefficients of images of cells loaded with Fura that were thresholded manually as compared to those using the MCT algorithm (Fig. 2E3, \( P = 0.303, N = 5 \), ANOVA), we did see that in some cases, the manual threshold chosen failed to identify features which, upon further investigation, we confirmed to be labeled cells. In this respect, the MCT algorithm mirrors, or improves on, the qualitative assessments made when thresholds are selected manually with the similarity in correlation coefficients, reflecting this equivalence (Fig. 2F, in vivo dendrites and axons \( R^2 = 0.78 \pm 0.16 \), LGN images \( R^2 = 0.92 \pm 0.03 \), calcium \( R^2 = 0.61 \pm 0.32 \), error = s.d. Fig. 2G). Linear regression \( R = 0.527, P = 0.0027 \).

4. Discussion

In summary, MCT is a powerful and easy to implement technique for rapid thresholding of biological data in vivo and in vitro. The algorithm successfully thresholds high bit depth imaging data such that the resultant binary image is highly similar to images generated by a user performing manual thresholding. Furthermore, MCT performs robustly even when images are noisy (Figs. S1 and S2) or median filtered (Fig. S3) and especially when the features of interest are sparse or faint. This improved performance is highlighted when we compared MCT to other automated thresholding algorithms (Fig. S4) and is faster than methods that use correlations for thresholding.

While this study has focused on the efficacy of MCT on image processing of sparse neuro-anatomical data, the simplicity of its use and the ease of its implementation make it well suited for all classes of binary global thresholding. Possible extensions present a number of novel approaches to image analysis. While we confined our analysis to global thresholding, variations could be used to select regions of interest and calculate MCT curves for subdomains, each having a different MCT curve max for thresholding. In addition, various threshold values could be selected based on the second derivative of the MCT curve, transforming the binary operation of MCT into a compression algorithm. In this regard, MCT provides a robust, reliable, and intuitive algorithm for general image processing.

Acknowledgements

We would like to thank Jennifer Dry-Henich for help with animal care and procedures, Dr. Robert Murphy for providing us with MATLAB code for the Ridler-Calvard algorithm. We also used an implementation acquired from the Mathworks central file exchange by Dharmesh Ramachandram. We also thank Dr. Roberto Fernandez Galan for helpful discussions and comments and Dr. Nathan Urban for helpful comments and for allowing us to perform calcium imaging experiments in his lab, Tranzyme Inc. and Dr. Pavel Osten for providing us with lentivirus constructs, and Dr.
Brooke McCartney and Dan Smith for providing us with images for thresholding. We also thank Mihaela Obreja for helpful comments on the manuscript. This work was funded in part by fellowships NSF DGE-9987588 (K.P.) and DGE-0549352 (K.P.) and support through a Searle Scholar Award (J.C.C.), an A.P. Sloan Research Fellowship (J.C.C.), the Dana Foundation (J.C.C.), and the Human Frontiers Science Program (J.C.C.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneumeth.2010.08.031.

References