Semi-Automated Cellular Tomogram Segmentation Workflow (CTSW): Towards an Automatic Target-Scoring System

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ABSTRACT

Electron tomography (ET) is a powerful tool for quantitatively mapping the complex 3D sub-cellular structures of cells. High accuracy segmentation results are great value to cell biologists. They facilitate the comparison processes across statistically significant datasets of properties or structure information like size/volume of cellular compartments. Manual segmentation is reasonably accurate but the process might be too slow – since the accuracy is highly dependent on the training of the person conducting the task. Automated segmentation therefore opens a number of opportunities. But these automated methods must be fast and capable of accurately delineating all contours of interest, ideally at organelle and molecular level – where many of which were reportedly not successful on ET datasets. Semi-automated approaches however have substantially allowed wider scope in resulting maintained cellular membrane tracing quality and accuracy and providing improved segmentation time. These reasons have motivated the development of a pipeline – semi-automated cellular tomogram segmentation workflow (CTSW) with particular components – that will find the best settings of chosen combination methods for high resolution tomogram segmentation specific to the intrinsic properties of the image volume being processed. The study also introduced a set of scoring objectives to enable timely segmentation of cellular compartments and expedite the process of optimizing method settings.

KEYWORDS

Image processing, watershed, image filter, cellular tomography, segmentation

1 INTRODUCTION

Image segmentation is defined as the mathematical process of separating or partitioning a digital image of an object (e.g. an organelle) from a larger, more complex image dataset into multiple ‘segments’ (i.e. sets of pixels) of non-overlapping, adjacent regions. These segments have some basic visual characteristics in common (i.e. intensity, color, texture) [1], [2] which when accurately defined, can provide a meaningful yet simplified representation of the information within the original image. Segmentation is thus a crucial step in defining basic structural features of cells and sub-cellular compartments, prior to quantitative analysis and annotation of complex cellular images.

Manual tracing is most widely used for segmenting complex structure resolved by electron tomography. Typically users experienced in the cellular and/or biological processes of interest must analyze each tomogram before the objects of interest (OOI) can be properly segmented. Dedicated software packages that provide manual contour drawing tools for biological image analysis include IMOD [3] and TomoJ [4]. Familiarization with the visual heuristics of the tomographic image data, aids the expert in recognizing cell organelles, thereby enabling them to carefully draw the contour lines defining each organelle boundary. The drawing process is then repeated on each adjacent slice of objects in the tomograms across every slice spanned in Z.
set of contours for each object are created prior to generating a continuous 3D surface where the triangular/polygonal meshes between adjacent contours belonging to the same surface are matched. The meshing process is carried out on each organelle to produce a high fidelity 3D model to describe the spatial and structural organization of compartments and other structures within a cellular region, and to compute precise quantitative data [5]. The drawback of manual segmentation of large cellular tomograms is that it is very labor intensive. As reported in [6] a tomogram estimated to represent just 1% of the total volume of a mammalian cell required approximately 3600 h (9-12 months) to completely segment manually at the organelle level. Furthermore differences in criteria applied by different experts/users can lead to differences in volume estimation of some cellular tomogram regions. The highest consistency and sensitivity of manual tracing is therefore achieved when a single individual traces the entire dataset. Achieving consistency in tracing such complex structures is also painstaking for the expert and time consuming. For these reasons, computational procedures for segmenting and quantifying the organelles have attracted considerable interest.

Segmentation of high-throughput cellular datasets, using a ‘one size fits all’ fully automated segmentation algorithm is complicated by the inherent structural diversity within various cellular compartments. Furthermore to achieve accurate 3D models of organelles for visualization and/or annotation purposes, particularly for complex organelle structures, the performance of fully automated segmentation algorithms (i.e. that use ‘one standard parameter setting’ or parameter free algorithm) may highly depends on post-processing. Post-processing at the final stage of organelle segmentation is always time consuming and less consistent in terms of accuracy [7], [8], [9]). The problems of manual and fully automated segmentation processes can be addressed through the development of semi-automated segmentation processes based on the careful definition of structural features of cellular compartments such as organelles.

The process of segmentation is greatly enhanced by first applying an image filter that ‘reduces the noise of inherently low signal: noise electron tomography data while preserving object edges. Several methods have been developed for reducing image noise either using general approaches such as image filters (e.g. rank filters, classical filters) or by focusing on a particular type(s) of noise ([10]. Many image filters have been developed to suppress background noise, such as low pass, Wavelet transforms and median filters [11]. Image filters that have been successfully applied on ET include median filters [12], [13], bilateral filter [14], [15], [16], [17], [18], and diffusion-based filters [19]. Nonetheless, the capability to suppress noise without blurring the high resolution details (the signal) remains the main challenge in image filtration processes and typically there it is a requirement to balance noise reduction with signal preservation.

The aim of this project is to design and develop a semi-automated workflow for cellular tomogram segmentation. The purpose of this cellular tomography segmentation workflow (CTSW) is to highlight feasible procedures for obtaining optimized settings for accurate organelle segmentation. Essentially a dataset containing sub-volumes representing Golgi apparatuses, mitochondria and insulin granules are classified into their types and a range of pre-filters and edge detection methods tested with the aim of developing a more automated process of image segmentation based on the concept that the properties of images in a given subset class (e.g. organelle complexity) will be relatively similar. Performance of the workflow is evaluated by comparison to the manually-segmented reference set.
2 MATERIALS AND METHODS

2.1 Organelle of Interest: Selection, Extraction and Manual Tracing

Membrane-bound organelles, the Golgi apparatus (GA), mitochondria (MC) and insulin granules (IG) were selected due to their biological importance in the insulin secretion process [20], [21], [22], [23], [24], [25], [26]. These key organelles have distinct, distinguishing image features and organelle structures [27], [28], [29], [30], [31], [32], which potentially make the segmentation technically challenging. Cellular compartments were extracted using the ‘newstack’ function in IMOD and the organelle was boxed out as a set of 2D slices. These sub-volumes were then classified into their organelle type (i.e. the Golgi apparatus, mitochondria and insulin granules). The membranes of the organelles were manually traced in order to obtain a control contour dataset. Manual tracing of the cell organelles was performed using IMOD Package Tools [3] in native space.

2.2 Manual Segmentation

Manual segmentation is usually considered the best and possibly the only practical approach for segmenting organelle contours of heavily noise contaminated cellular tomograms [30], and hence was used to establish a reference set (or control dataset) to validate the performance of computational workflow segmentation results. For this purpose, organelle membranes were manually traced to produce sets of contours using the drawing tools in IMOD. Sets of contours of every sub-volume were then modelled (using imodauto function in IMOD) and meshed (using imodmesh function in IMOD).

2.3 Designing a Workflow for Cellular Tomography Segmentation

To design and develop the CTSW, a series of studies were conducted on two different image pre-filtering methods; 3D Median filter [13] and Non-linear Anisotropic Diffusion (NAD) [33], [34] filter and region-based segmentation algorithm (Watershed algorithm) to evaluate their benefits and limitations for the proposed study. Based on these results the CTSW was developed and the performance of this validated against the manual tracing method.

2.3.1 Processed image for accurate contour tracing

Pre-filtering is an effective way to denoise the images prior to segmentation – in particular 3D median filter and non-linear anisotropic diffusion filter [10]. These two pre-filters were proven to be applicable for denoising cellular tomography [10], [13]. Based on the method descriptions, they could significantly improve the signal-to-noise ratios (SNR) of these ET images prior to organelle segmentation, thereby improving the quality of the latter. Their effectiveness was expected to depend largely on the image characteristics. In this experiment, the recommended parameter settings (RPS) – i.e. optimized setting – of respective image filters were used (Table 1).

<table>
<thead>
<tr>
<th>Image filter</th>
<th>Software Package</th>
<th>RPS (initial settings)</th>
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<tbody>
<tr>
<td>F1: 3D Median</td>
<td>CoAn</td>
<td>Kernel size: 3x3x3, Iteration: 3</td>
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<tr>
<td>F2: NAD</td>
<td>IMOD</td>
<td>K value: 5.6, Iteration: 20</td>
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Table 1. Pre-filtering approaches and their respective initial settings (i.e. proposed by the software/method developer), F1: 3D Median filter, F2: Non-linear anisotropic diffusion filter, RPS: recommended parameter setting.
2.3.2 Watershed Algorithm

The Watershed transform [2] that was developed for accurate biological specimen segmentation has numerously used for various image segmentation, and thus was selected to be used in this experiment. This automated region-based segmentation has technically proven its ability in detecting object regions based on region’s intensity. The method accurately detected the contour of interest (i.e. the organelle’s membrane) including those ‘unwanted neighboring contours’ (Figure 1).

Using optimized settings of the watershed transform, the segmentation result of insulin granule yielded a clear separation between the contour of interest (granule’s membrane) and the contour of ‘neighboring’ objects. Similarly, the most complex of the three key organelles, the Golgi apparatus, yielded good tracing result for the majority of the combination approaches (Figure 2).

The simplicity of the IG appearance particularly the immature granules (i.e. a single layer membrane, smaller in volume than neighboring objects/organelles attached or close to organelle membrane have made the automated segmentation easy and straightforward (Figure 3 A). While the most complex organelle amongst three key organelles, i.e. the Golgi apparatus, also showed promising results. Although there are problems such as over- and/or under- segmentation, the contours of cisternae membranes of various morphologies were successfully traced by the optimized automated settings. Mitochondria on the other hand, could not be segmented with the initial settings (Figure 3 B). Closer analysis showed that the double-layered organelle is denser than other organelles and neighboring objects and this appeared to directly influence the segmentation process and optimal settings required. In particular the use of the ‘invert’ function improved segmentation quality. Even though watershed obtained accurate tracing results, unwanted objects remain the main drawback for automated tracing. These required subsequent removal. As the project’s prime objective is to produce a rapid and accurate segmentation approach, computational approaches were sought to remove unwanted contours. Prior to this, edge or contour refinement is essential to improve the traced contours and visualization.

![Figure 1. Watershed transform applied on optimised filtered image (insulin granule). (A) A labelmap image with rough spatial shape has been transformed to a smoother shape (B). Every single object in (B) is traced (C). For accurate membrane contouring, the organelle of interest is highlighted in light blue (D) where the light blue contour is disconnected to neighbouring contours (green). PT: ~20 s, SA: ~8x10^4 nm². The ground truth dataset of this example; PT: 600 s, SA: ~5x10^4 nm².](image1)

![Figure 2. Majority of the tracing results show main Golgi structures (i.e. the cisternae) are traced, with a slight over- and under-segmentation. Images represent the traced contours laid over real dataset of the GA images. A: Optimised 3D Median filter followed by watershed transform. B: Optimised NAD filter followed by watershed transform.](image2)
Figure 3. Two different organelle, insulin granule (A) and mitochondria (B) were manually traced. Both examples were also segmented using the recommended parameter setting of Method 1 (M1). This setting successfully traced the insulin granule membrane (A). However, it failed to draw the contour of mitochondrion membrane (instead it traced the contour of other objects). ‘Invert’ (parameter in watershed segmentation) is turned on in the second trial and mitochondrion membrane is finally traced. M5 - i (n): number of iteration, watershed - HC: high-contour cut-off, LC: low-contour cut-off, I: inversion contrast, mathematical morphology – I: initial opening, O: opening, C: closing.

2.3.3 Contour line refinement: Mathematical morphologies algorithm

Contour refinement allows ‘apparent separation’ between the contour of interest (Figure 4) and unwanted contours. Practically this can be done using a mathematical morphology algorithm. There are two operations in mathematical morphology commonly used for contour refinement. These are opening (i.e. erosion followed by dilation operation) and closing (i.e. a dilation followed by erosion operation) [35]. The maplmorph function in CoAn [36] was used for this study. It detects edges and disconnects objects using optimized settings of opening and closing (Figure 4). This will allow the object of interest to be selected computationally (Figure 5).

2.3.4 Meshing: Contour volume value and mesh surface area scoring

Based on a 3D mesh contour of an object, its volume (CV – contour volume) and surface area (MSA – Mesh surface area) can be calculated and used for quantitative comparison with the manual reference set. Meshing was performed automatically by using the imodmesh function in IMOD. The process was performed on the segmented stacks of image slices of each organelle. The contour volume (CV) is a sum of the area of contours (of each of Z slice of an object) times the distance to the connected contours in Z; The CV value is chosen for two reasons; 1) it handles the problem of skipped sections [3]; and 2) it gives a slightly more accurate volume measurement for the capped regions because it integrates with a trapezoidal approximation [3]. The mesh surface area (MSA) is the total surface area of a mesh volume, computed by adding the areas of all the triangles in the mesh.

In theory an object accurately segmented by two independent methods should have identical contour volumes. To test the effectiveness of contour volume (CV) as a metric to quantitatively compare contour sets of automated segmentation, they were assessed in comparison to a manual reference. By doing this, unwanted contour sets could be deleted in an automated manner when the CV value of automated segmentation did not closely match the CV of manually traced referenced contour sets. Furthermore this approach proved useful when more than one contoured volume was traced in a given tomographic sub-volume as...
the \textit{mappick} function (\textit{CoAn}) can be used to detect and delete unmatched volume(s). MSA, provide an exact measure of the area of the mesh, and was used as the second assessment of the segmentation results and for identification of the best combination of method settings.

\textbf{2.3.5 Proposed method flow for automated and accurate cellular compartment segmentation}

The proposed workflow (Figure 6) is based on four main stages; 1) data preparation (e.g. sub-volume extraction, denoising), 2) segmentation, 3) statistical evaluation, and 4) contour selection. All filtered stacks of images were then segmented using the watershed function (i.e. \textit{mapcarv}), provided by \textit{CoAn} [36] to produce a label map. The \textit{CoAn} system automatically checks these label maps for unwanted contours which usually appear ‘smaller’ than the object of interest – by turning on ‘deleting blobs’ function in \textit{CoAn}. Function \textit{mappick} (\textit{CoAn}) was used to delete all these unwanted densities. Next mathematical morphology algorithm (i.e. \textit{maplmorph}) was applied. Lastly a set of contours for every stack was produced. The process of optimizing parameter settings particularly for image filtrations and watershed transform was started using the recommended settings of the respective software developers. The ‘ground truth’ datasets were generated from manual tracing of the datasets. From these contour sets, cylinder volumes (CV) were calculated by taking the area of each contour times the thickness of the sections (defined by pixel size and Z-scale) [3], summed over all of the contours (\textit{imodinfo}). Stacks of contours of each sub-volume were meshed, and meshed surface area (MSA) of each was calculated and saved. The CV information of respective ground truth datasets is important to identify optimal settings of the CTS method’s parameter(s). The MSA information was used to validate the best (optimized) method flow(s) amongst the two method flows for particular cases of organelle segmentations (i.e. both calculations; CV and MSA are important and are used to stop the parameter adjustment process). The ‘best’ setting of the method was determined when the segmentation result met these three requirements; 1) the membrane contour(s) of the targeted organelle was/were segmented, 2)
no additional unwanted contours of neighboring objects or organelles were segmented, 3) once requirement (1) and (2) were achieved, the mesh surface area (MSA) of the traced contour(s) was calculated to establish whether it was within -/+ 25% (Figure 7) of the MSA calculated for the manually segmented sub-volume. The most critical and time consuming procedure is the second one (i.e. parameter settings optimization) as it employs a ‘trial and error’ routine. Consequently a ‘target scoring system’ was proposed to help identify the ‘best’ combination of parameter settings of the different method flows (Figure 8).

### 2.4 Refining parameter settings of segmentation method flows

To refine methods capable of more automated approaches to segmentation, sub-volumes of each of the three key organelles were randomly chosen such that a variety of sub-volumes sizes were represented for each organelle. The recommended parameter settings (RPS) of both method flows were employed on these key organelles sub-volumes prior to optimizing the methods settings. Contour sets were meshed.

These meshed volumes were then used to compute the CV and MSA. By using the CV, unwanted contours or contour volumes (i.e. which are not similar to the CV of the ground truth) were deleted computationally. Optimization of settings was continued until the MSA of the computational method approached the values of the manual MSA of the ground truth set (a score of 5 being best and 1 the worst). Insulin granules and particularly immature granules with a ‘simple appearance of structure’ (i.e. empty lumen and smooth boundary membrane) were successfully contoured (score 5) with optimized image filters and optimized watershed of every method flow (M1 and M2).

![Figure 6. Workflow diagram of the proposed systematic approach for accurate segmentation.](image-url)
Figure 7. Target scoring system where ‘100%’ is referred as a ‘target point’ of ground truth datasets. All mesh volumes of manual tracing is ‘labelled’ as 100%. Any results of computational methods scored +/- 100% (of respective MSA) will determine a score of 5, and so on. Target scoring for each sub-group will differ according to its ground truth (gold standard) datasets. 5 scores of MSA range were proposed where ‘5’ is the best while ‘1’ is the worst.

Figure 8. (A) Manual segmentation (red contour) of the raw data. The cyan contours in B to D are the results of ‘best’ settings of 3 different method flows that are superimposed on raw data. (B) M1 (optimised 3D Median filter followed by optimised watershed and mathematical morphologies) where the MSA was ~91% of the MSA manual segmentation (i.e. the MSA of M1 was less ~10% from the MSA of manual segmentation). This result scores 5. (C) M2 (optimised non-linear anisotropic diffusion filter followed by optimised watershed and mathematical morphologies), the MSA was 101% and this was the most accurate result of the method flows tested for this mitochondrion segmentation. This result also scores 5. Scale bars: 100 nm.

The CV and MSA value of these example datasets consistently achieved a score of 5 according to the ‘target scoring system’. On the other hand, almost all sub-volumes of the Golgi apparatus faced similar problems when segmented, exhibiting both over- and/or under-segmentation (Figure 9 A). Because of this common problem, optimizing parameter for the Golgi segmentation took longer time as compared to insulin granules. Setting optimization was commenced with image filtration followed by the watershed (Figure 9 B).

2.5 Quantitative and qualitative analyses

For the best analysis process of segmented result and to choose the best parameter settings of particular organelle sub-groups, quantitative and qualitative analyses were established accordingly. Number of contours (NOC) was first manually examined. The closest NOC value of automated tracing to respective NOC obtained manually will be chosen. Results with ‘less or free roughness’ were visually selected for the next assessment, i.e. using scoring system. Respective optimized settings of the best segmentation result (or the best three) were recorded. These settings were used to segment other organelle sub-volumes of the same organelle type. Parameter adjustment was performed for accurate tracing results of respective organelle sub-volumes.

Figure 9. (A) Segmentation result of the proposed settings shows smooth segmented contours, however it is lacking in number of segmented cisternae. RPS: Image filter: Median 3D: 3x3x3, i(n): 3, Watershed HC:1.0, LC: 0.0. (B) The OPS of [M2] shows a better segmentation result where more contours of the cisternae was segmented, without adjusting the setting of watershed. OPS: Image filter: Median 3D: 3x3x3, i(n): 5, Watershed HC:1.0 LC: 0.0. Median 3D i(n): Number of iteration, Watershed HC: high contour cut-off, LC: low contour cut-off. RPS: Recommended parameter setting. OPS: Optimised parameter setting.
3 DISCUSSIONS

Pre-processing is an essential step for accurate segmentation. It is necessary to suppress image noise as well as enhance the organelle edges. There are many demonstrated pre-filtering methods capable of improving the signal-to-noise ratio of an image and enhancing edges of the objects or cellular compartment and suppress the noise to facilitate the tracing process [10], [13]. However, most of them obtain their best capacity if the ‘noise’ is known [37], [38], [39], [40]. To date, no single settings of mathematical segmentation method has shown its effectiveness on whole tomograms, i.e. so that every single cellular compartment is accurately traced without any ‘extra segmented regions/contours’.

To establish the final segmentation results for better visualization and to benefit the analyses of results, an effective pipeline of automated segmentation for accurate segmentation result was developed. The pipeline involves three main stages; 1) data preparation including sub-volume extraction and image file conversion, 2) optimized parameter settings which involve three main steps in automated segmentation including image filtration, watershed algorithm and mathematical morphologies operations, and 3) statistical evaluation using target-scoring system that is used to identify the ‘best’ combination segmentation method amongst two different method flows. Contour selection will be applied when the deletion of unwanted contours are needed.

Despite specific abilities in suppressing background noise, or improving edges of targeted membrane organelle, the settings of parameters for particular method flow were defined as the best (optimized) based on three primary analyses including visual observation and mathematical computation.

Even though this research has reported a proposed segmentation pipeline that has given promising computational results and increase automated segmentation efficiency, the identification of optimal setting for 400 sub-volumes is still time consuming. It will be harder for 4000 or even 40,000 sub-volumes. It was also found that mitochondria are radically ‘darker’ (high contrast image organelle) than the insulin granules and the Golgi apparatus the settings of watershed were run using ‘invert’.

As image contrast was found to be important for accurate automated segmentation, classification of similar objects into separate classes could facilitate contouring process. It was hypothesized that additional image properties for each key organelle could expedite the identification of optimal image processing settings within the method flows, and thus further ‘simplify’ the process, with the potential to identify standard optimized settings for organelle segmentation according to its image properties.

REFERENCES


