A novel 2D and 3D method for automated insulin granule measurement and its application in assessing accepted preparation methods for electron microscopy

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Abstract. Transmission electron microscopy images of insulin-producing beta cells in the islets of Langerhans contain many complex structures, making it difficult to accurately segment insulin granules. Furthermore the appearance of the granules and surrounding halo and limiting membrane can vary enormously depending on the methods used for sample preparation. An automated method has been developed using active contours to segment the insulin core initially and then expand to segment the halos [1]. The method has been validated against manual measurements and also yields higher accuracy than other automated methods [2]. It has then been extended to three dimensions to analyse a tomographic reconstruction from a thick section of the same material. The final step has been to produce a GUI and use the automated process to compare a number of different electron microscopy preparation protocols including chemical fixation (where many of halos are often distended) and to explore the many subtleties of high pressure freezing (where the halos are often minimal, [3]).

1. Introduction

Insulin is secreted into the blood stream by beta cells inside Islets of Langerhans of the pancreas. After its biosynthesis, insulin is processed and packaged in granules, surrounded by a membrane. Quantitative measurements of the amount of insulin in the cell have physiological importance, so it is important to be able to take measurements from images, ideally in an automated way. In TEM the granules usually appear as dense cores often surrounded by a halo and enclosing membrane. However the sample preparation route can strongly affect the appearance, such that the cores vary in intensity and texture, (and so can be confused with other cellular organelles such as mitochondria), and some granules have no surrounding halo or only partially visible membranes (Figure 1). All of these factors make accurately segmenting granules a challenging task. We have developed a novel method based on active contours which we have shown to be superior to other mathematical methods previously explored [1] and does not require an expensive software package to run [2]. Indeed we have now built a (soon to be) freely available GUI which runs through MatLab. We first validated our algorithm

against manual measurements. Per image, the automated method was not substantially faster but it was then applied to a large dataset where samples were prepared in the same laboratory for electron microscopy using a large set of different sample preparation protocols. For this analysis we have measured in excess of 10,000 granules and have found that there are clear differences between chemically fixed (CF) and high pressure frozen (HPF) samples, and also subtle variations depending on the post-HPF freeze substitution recipes. Furthermore, in order to achieve true volume measurements we have extended the method to 3- dimensions (3D) by applying it to an image z-stack from a tomographic reconstruction dataset.
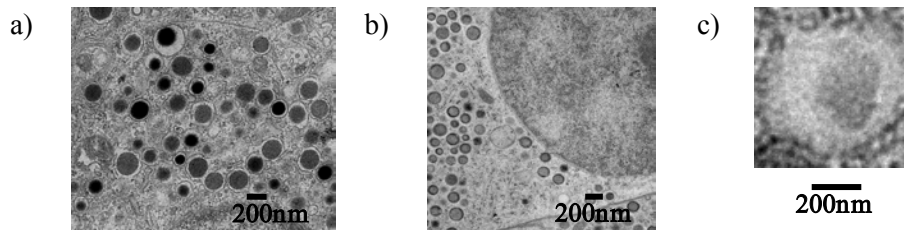

Figure 1. Varying appearance of secretory granules a) CF b) HPF c) Incomplete membrane

2. General description of the algorithms used for core and halo segmentation

2.1 Core segmentation
Due to the complexity of the image, the core segmentation process is broken down into 3 steps (Figure 2).The first step uses a locally scalable region-based level-set active contour with a shape reguliser to provide a good pre-segmentation of the cores. Other unwanted cellular components segmented at this stage, are discarded if they are not round enough or are too large. The cores are then accurately segmented by using a dual level-set active contour. The algorithms have been published elsewhere [1].
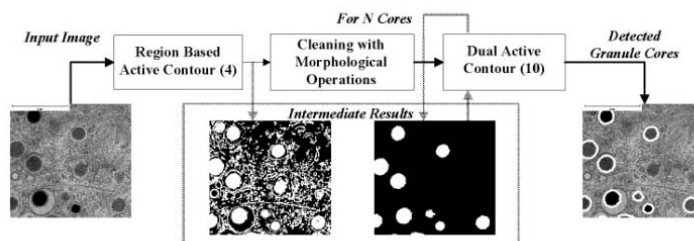

Figure 2: Granule core detection algorithm using section of TEM image 200 x 200 pixels (pixel size is 5nm)

2.2 Segmentation of granule membranes
The initial active contour segmentation result (segmented cores), and the original TEM image are starting points for membrane segmentation. The core image is slightly dilated and then removed from the image. The granule membrane is then sampled by projecting outwards from the core in all directions until the first non zero pixel is found. Morphological operations, sampling and application of a vector field convolution snake, are needed to fill gaps in incomplete membranes. The application of a further sliding band convergence filter is used to verify when cores have no halo which is often the case for the HPF samples. The work flow is shown in Figure 3.The mathematical equations were presented elsewhere [4].

2.3 Extension to 3D
The beta cell tomogram (z-stack) slice images are quite different from their conventional 2D counterparts as they are reconstructed from a series of tilt images from a 300nm section and also the reconstruction algorithm plays a part. We selected the most commonly used weighted back projection in the IMOD software package [5]. This leads to an image with relatively poor contrast when

compared to the conventional images from 70nm sections. The poor contrast has the greatest effect on the appearance of the granule halos. An initial segmentation is first performed on each slice to get a good estimate of the granule core locations; a second refining segmentation is then done on each granule using a dual region-based active surface, first for the core. For the halo we use a dual Bayesian active surface. A novel boundary prior and gradient term have been incorporated in a dual active surface, to address the challenges faced with halo segmentation. This method has been shown to be superior to other active surface models [6]. We show a completely encapsulated granule core and halo in Figure 4 manually segmented in (a) and (c) and using our algorithms in (b) and (d). Note that since the granule is segmented in each slice it does not look completely smooth in the vertical plane.
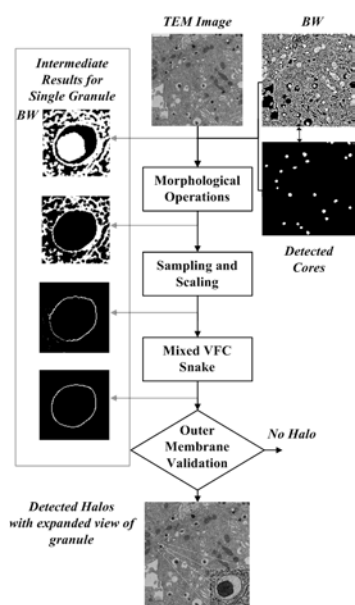

Figure 3: Membrane segmentation algorithm
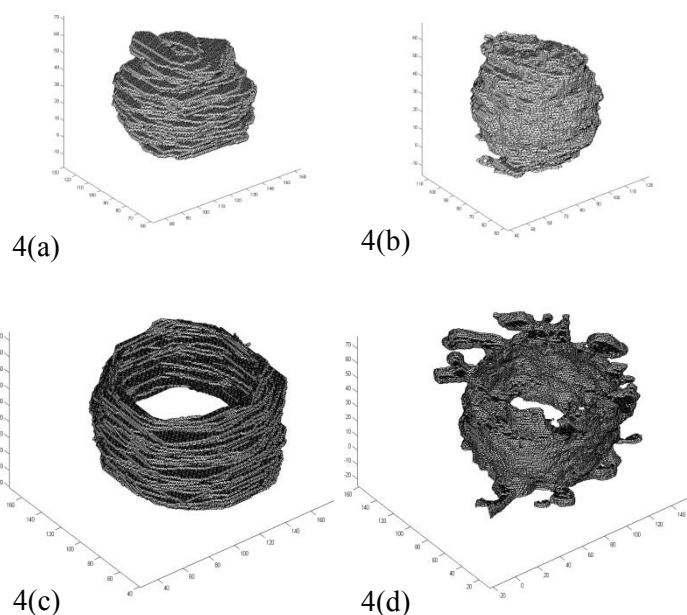

4(a)   4(b)

4(c)   4(d)

Figure 4: 3D core (a,b) and halo (c,d) segmentation. 4(a) and (c) done manually, (b) and (d) with our algorithm. Dimensions in pixels where pixel size = 3.8nm

3. GUI

The user interface is currently being tested and an example of the page for complete segmentation is shown in Figure 5
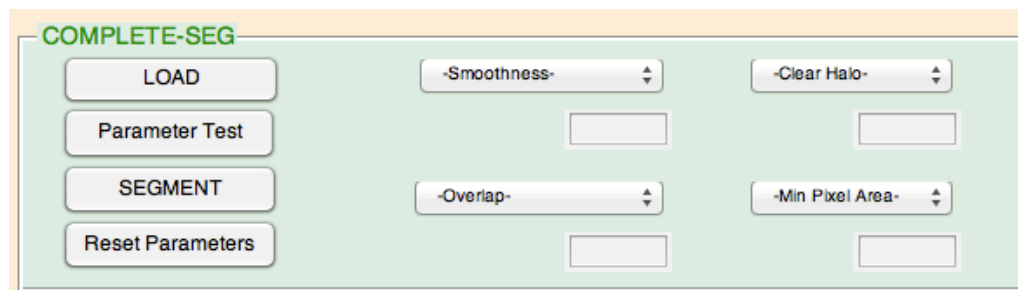

Figure 5: The Graphical User Interface

After loading an image, a ROI is selected and user selectable parameters for effective segmentation can be input and evaluated. The values of the parameters depend on the character of the image and sample preparation method. "Smoothness" encourages granule circularity, for example. "Clear halo" removes contrast features between the granule and the membrane and "overlap" accounts for the minimum overlap of the segmented (possible) membrane and the initial segmentation. Guidance on appropriate values is given in a Help function. A test segmentation on the ROI can be performed and

evaluated before committing to the complete segmentation. The results are output to Excel spreadsheets. A batch programme is also available to handle many images for say, overnight analysis.

4. Experimental Results

4.1 Validation of algorithms

The algorithm was initially applied to seven 3x3 tiled images each showing a complete beta cell prepared either by CF or HPF methods. The manual measurements made on the same images were considered to be "ground truth". The number of cores detected was within 96% of the number of cores counted manually. We also compared average core and membrane areas, with those measured manually. The metric we used to record granule sizes is the radius of a circle of the same area. On average the granule core radii difference for all images is 13.35% and for membranes 6.08%.

The analysis of the z-stack in the tomograms is in its early stages. The visual inspection of post processed granules, as in Figure 4, looks promising. The process compares with manually segmenting and building a model to generate volume data as with IMOD software, which is extremely labour intensive. Thus to be able to extend volume measurement to thousands of granules would only be realistic with automated software such as ours.

4.2 Comparison of Specimen preparation protocols

10 (4k x4k) EM images were taken from each of sixteen differently prepared samples and these were analysed using our software. The core and membrane equivalent radii for each granule were taken as the dependent variables and the fixation method as the independent variable. A MANOVA indicated that the fixation method does indeed affect both the core and membrane areas. An ANOVA test then confirmed that there is a significant difference in mean areas for both cores and membranes between the different sample preparation procedures. Tukey's HDS test was used to do multiple comparisons. We found that for our standard CF method (fixed with 2.5% glutaraldehyde in cacodylate buffer), there is a significant difference in membrane areas when compared with all other methods. For our standard HPF method (freeze substitution time 5hours (-90°C to 0°C), with 1% osmium + 0.1% uranyl acetate in acetone)[3], there was a significant difference in membrane areas when compared to the CF methods, but no significant difference in membrane areas when compared to most of the other freeze substitution methods. Detailed evaluation of the differences between the 16 methods is currently being done.

5. Conclusions

We have developed automated software using active contours to segment insulin granules more effectively than other published methods. A GUI has been designed to allow non experts to use the programs to analyse images. The algorithms have been extended to analyse 3D tomographic z-stack data. Ultimately a measure of the volume of insulin per cell or differences between healthy and diabetic animals will provide most useful physiological information. Furthermore the results are allowing a meaningful comparison of published EM sample preparation protocols.

References

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