The paper is organized as follows: in section 2, the context of the study is described; in section 3, the specimen preparation and image acquisition methods are briefly described; in section 4, the approach for membrane segmentation and its implementation are depicted; finally, before concluding, the results are discussed in section 5.

2. BACKGROUND

2.1. Biological Context

Proteins are part of all living organisms and understanding their functions is a big challenge [1]. Membrane proteins represent about 30% of all proteins. Although the structure of more than 40 thousands of proteins has been solved, only a few hundred membrane proteins have been determined, according to S.H. White’s Website [2] and the RCSB Protein Data Bank [3]. Most of the proteins have been solved by X-Ray diffraction of 3D crystals. It requires large amount of soluble proteins devoid of lipids, which makes this method inappropriate for most membrane proteins. A good alternative for the analysis of membrane proteins is the 2D-crystallization method that arranges the proteins into lipid layers (artificial membranes). However, lots of trials are needed to determine the required crystallization conditions for a given type of proteins. Thus, it is worth developing a tool that can routinely assess the results and the quality of the crystals for the numerous tests.

2.2. Microscope Control and Specimen Assessment

Some efforts have been placed on the automatic screening of samples with a TEM [4, 5], but the required automatic analysis of the images appears to be still in its infancy.

In this project, the quality of the crystals can be assessed in the frequency domain at high magnification (about x50,000). Since the membranes on the 3 mm diameter grid can be sparse, screening at that magnification is inefficient. Thus, a preliminary screening is done at a medium magnification of about x5,000 to localize the membranes, pre-analyze them and identify the regions of interest (ROI) to be examined at high magnification. This pre-analysis also aims to classify the objects by an evaluation of the aggregation of the membranes, their shapes and sizes.

The ROI are large non-aggregated sheet-like membranes. They are especially difficult to identify for several reasons. Firstly, the size and shape vary greatly and the identification cannot rely on pattern recognition methods. Then, though the negative staining tends to locally enhance the contrast, the grey level of the ROI is still very low compared to the aggregates and not much higher than the level of the background and the noise (Figure 1), which makes simple thresholding segmentation improper. Though gradient-based methods are more suitable, simple edge detection is not sufficient. The choice of the kernel size of the filter is difficult because of the noise, the various width and contrasts of the edges. The obtained gradient images can then be segmented by thresholding or by applying the...
watershed method. The selection of the threshold value is however critical: either all the true edges are selected, but there are also many faulty edges due to the noise, or the noise is removed but the edges are incomplete. The main advantage of the watershed method is to result in closed regions. However, this algorithm is very noise sensitive. The proposed method combined the advantages of both thresholding and watershed: the threshold is used to reduce the noise of the edges detected at several scales and the watershed algorithm is applied to the RGL image.

This paper focuses on the segmentation of the images at medium magnification. This step leads to a segmentation of the image into uniform regions that will characterize the sample and identify the areas of the membranes (ROI defined above) that are the most suitable for high magnification analysis.

3. SAMPLE PREPARATION AND IMAGE ACQUISITION

The specimens have been prepared as explained in [6]. They have been placed on carbon coated copper grids commonly used in TEM. Then, samples have been negatively stained with uranyl acetate to enhance the contrast of the biological samples. As a drawback, staining generates artifacts and locally heterogeneous backgrounds.

The algorithms have been developed with images acquired using different microscopes at medium magnification of about x5,000.

4. MEMBRANE SEGMENTATION

The proposed method (Figure 2) for membrane segmentation generates a RGL image that will be treated with the watershed algorithm. The multiresolution approach is used in a specific way by combining the edges detected after a scale-adapted threshold. As the scale gets coarser, more edges can be detected because smoothing does not have an even impact on all image pixels.

4.1. General Multiresolution Approach

Multiresolution methods are transformations to obtain an image at different scales. The general concept relies on the fact that some details can only be observed at certain scales, handling the problem of filter kernel size. The structures at the coarser scales are simplifications of those at the finer scales by low-pass filtering, and it is generally considered that no new structures are created in the transformation from fine to coarse scales [7, 8]. From Lindeberg [9], the scale-space representation of $L : \mathbb{Z}^2 \times \mathbb{R}_+ \rightarrow \mathbb{R}$ can be defined for a 2-dimensional discrete signal $f : \mathbb{Z}^2 \rightarrow \mathbb{R}$ as:

$$L(x, y, 0) = f(x, y)$$

(1)

at scale $s=0$ (initial image) and, at coarser scales ($s > 0$):

$$L(x, y, s) = \sum_{m=-s}^{s} \sum_{n=-s}^{s} T(m, n, s). f(x-m, y-n)$$

(2)

where $T(m,n,s)$ is a family of kernels of size $(2 \times k + 1)$, generally Gaussian kernels. The main features are selected at the coarser scales and then used in the finest scale to detect the details more precisely. Examples of this approach are given in [10, 11] to enhance the performances of the watershed algorithm and in [12] to detect the edges.

At fine scales, features are detected with precision but with many false edges due to noise. At coarse scales, noise is reduced but edges are blurred and detected with less precision [7, 8]. Therefore, the edges detected at coarse scales need to be tracked back to the finest scale to adjust their position.

Edges can be precisely detected with the zero-crossing of the second derivative analysis. Applied to our images, the study of the zero-crossing of the second derivative did not turn out to be effective, mainly due to its noise-sensitivity, and to maxima shifting through the scales.

4.2. Proposed Approach

The zero-crossing analysis considers only the local maxima of the first derivative. In this work, the evolution of the amplitude of the gradient is considered rather than studying only the position of the local maxima of the gradient. In accordance with the observations on our TEM images, since an edge pixel is to be less smoothed than a non-edge pixel, there exists a coarser resolution where the gradient of the edge is higher than the gradient of the noise. We observe that the gradient of many edge pixels $p_e$ is almost similar to the gradient of the background noise pixels $p_n$, and they cannot be dissociated by a threshold. However, a coarser scale will certainly exist where edges are enhanced from the

Figure 2 General diagram of the segmentation process with an example on the right column
The gradient of the edge pixels is less affected by the smoothing and there exists a scale where their value is over the threshold. Since this scale may be different for each edge pixel, it is important to search for the appropriate scale. Some approaches have been proposed to search for the best scales [13]. In TEM images, for a better compromise between edge detection and precision of their positioning, edges found at each scale are combined to obtain the RGL image with almost no noise (Figure 5 left). When the different binary images are combined, this compromise is handled by a weighted sum of the images: edges detected at scale $s$ are weighted by $(n-s)$, $n$ being the number of scales used. Thus, the combination results in the RGL image where the grey level is proportional to the precision of the edge detected. The weighted sum combination gives more importance to edges detected at the lower scales. The watershed algorithm is finally applied to the RGL image (Figure 5 right) where the edges are placed on the crests of the closed objects. Therefore, the edges are detected at the finest possible resolution.

This principle is now used to segment our TEM images into homogeneous regions.

4.3. Implementation

In this section, the implementation of the multiresolution transformation and the scale-adapted thresholding (first stages of the Figure 2) are described.

The multiresolution transformation can be implemented in different ways: either by reducing the size of the image (Gaussian pyramids, wavelets transforms...) or by increasing the kernel of a smoothing filter (usually the Gaussian kernel). For computational time reasons, a pyramidal transform is used. Dyadic pyramids reduce the size of the image by a factor of 2 between each scale. Some features may however be better represented and identified at intermediate scales and for this reason a non-dyadic pyramid obtained by interpolation has been favored.

For the implementation of the edge detection, a simple isotropic transform based on the 5x5 neighborhood standard deviation filter is suitable (Figure 4). An automatic detection of the threshold has been adapted to our types of images. The shape of the histogram of the gradient-images is mostly exponential-like and the ‘optimal’ point for segmentation was initially found experimentally to remove the noise at each scale; this threshold is located after the peak representing the lower gradient pixels (non-edge pixels or low-contrasted edges). Two methods can be used to automatically find this point: the triangle method from Zack et al. [14] or by a statistical estimation from Voorhees and Poggio [15]. The triangle is constructed by drawing a line $Lt$ between the maximum $N_{max}$ of the histogram and the highest grey level value $G_{max}$ in the image (Figure 6). The distance $d$ between the line $Lt$ and the histogram is computed for each bin. The threshold is chosen when $d$ is maximal after the peak. The statistical estimation relies on the hypothesis that the image is corrupted with white Gaussian noise. Voorhees and Poggio [15] showed that the magnitude of the gradient distribution is a Rayleigh distribution with a maximum

\[ G = \frac{p_e - p_n}{p_n} \]

noise thanks to the neighborhood weight. Hence, a threshold can isolate $p_e$ from $p_n$. This is illustrated on Figure 3 where 20 points of nearly identical gradient at scale 0 have been selected on an image and manually labeled as ‘edge’ and ‘non edge’ pixels. The average value of the non-edge pixels is generally decreasing. The threshold used to remove the noise can now be adapted at each scale to keep it optimal.

\[ \text{Threshold} = \frac{1}{n} \sum_{i=1}^{n} G_i \]

\[ G_i = \sqrt{G_{x_i}^2 + G_{y_i}^2} \]

Figure 3 Evolution of the gradient of non-edge pixels (left) and edge pixels (right) with the scale increasing (for 20 points of identical gradient at scale 0).

Figure 4 Example of edge detection at scale 2 and 4, for threshold automatically found at 9,000 and 6,700 (from Figure 1).

Figure 5 Based on Figure 1 and Figure 4, combination of the edges found from scales 1 to 4 (left) and regions found after the watershed algorithm (right).

Figure 6 Illustration of the triangle method on the histogram of a gradient image for the automatic determination of the threshold.
value at $N_{\text{max}}$. Under these assumptions, edges contribute mainly to the tail of the distribution and the location of the peak of the histogram is therefore mostly due to the noise. As a result, for an acceptable proportion $P_F$ of false edges, they proposed to fix the threshold $T$ of the gradient image with:

$$T = N_{\text{max}} \sqrt{-2 \ln(P_F)}.$$  

(3)

For example, to remove the noise with a confidence of 99%, ($P_F = 0.01$), then $T = 3 \times N_{\text{max}}$. For this threshold, we notice that in many images, the triangle method is almost equivalent to the statistical approach (see example on Figure 7).

5. RESULTS AND DISCUSSION

The process was evaluated on many images acquired with different microscopes: FEI Tecnai F30, Philips CM200, Hitachi H-7000 and H-8000. Images have been acquired with CCD cameras or scanned from photographic films. At a magnification of about 5,000, the goal is to characterize the sample and identify the ROI for high magnification image acquisition. As shown on Figure 5 (right), the method leads to results that are encouraging for this analysis step. The more aggregated areas are dark and often small regions. The ROI that are interesting to select are sheet-like membranes. Figure 5 and Figure 8 show the identified ROI whose boundaries are well-located. The areas of membranes form uniform and distinct regions (Arrows on Figure 8). Some edges are not step-wise but much more contrasted than the membrane itself (dotted ellipses on Figure 8), and undesired regions appear in segmentation. These regions are however relatively small and rare.

The current method relies on a set of scales and does not require looking for a unique optimal scale. Compared to non-multiresolution methods on these TEM images, the problem of the kernel size is controlled and there is a gain in the quantity of edges found. The watershed algorithm is here used to detect the closed homogeneous regions and gives better results than when it is applied directly to a smoothed gradient membrane image. The non-closed regions will simply be ignored (Figure 8, left column), but the chance of having closed regions is reduced when the number of scales used is increased (Figure 8, right column).

The number of closed regions identified is related to the number of scales used: using many scales would increase the probability of closed regions (Figure 8, right column). However, at coarser scales, the blurring is such that the edges are getting wider and objects can merge to form false crests and new local minima. It all means that the number of scales used must be well chosen to obtain a good compromise between having closed regions and avoiding false edges. We experimentally found for our $1k \times 1k$ images that the pyramidal transform should use between 8 and 10 scales (the $n^a$ scales being $1/n$ the size of the initial picture). The 10-scale implementation is preferred since a slight over-segmentation could be tackled by fusion during the labeling and analysis processes.

The choice of the threshold value also slightly influences the result of the segmentation, even if its influence is reduced by the multiresolution approach: if it is too high, the number of detected edges and the probability of having closed regions decreases; if it is too low, the resulting image is noisier, resulting in the identification of false edges and an over-segmentation of the image. The determination of the automatic threshold was studied from equation (3) by adjusting $P_F$. The compromise was experimentally found to better when the threshold is around $T = 2 \times N_{\text{max}} (P_F \approx 0.13)$.

Figure 7 Evolution of the automatic threshold over the scales with the triangle method (plain line) and the statistical method (doted line) with $T = 3 \times N_{\text{max}}$ for a given image.

Figure 8 Line 1: image initial; Line 2: RGL image after recombination of the multiresolution binary images; Line 3: Identified regions after the watershed algorithm; Segmentation with up to 8 scales on the left and up to 10 scales on the right. Arrows indicate ROI possibly crystalline. Dotted ellipse shows peak-like edges.
As it has been stated, because of the smoothing and the automatic thresholding, the edges are mainly becoming wider as the scale is increased, and the resulting drawback is that it may display unwanted crests and local minima that generate some false positive edges. Nevertheless, those are relatively rare.

According to the evaluation of a large number of images, the position of the true positive edges can be considered as equivalent to a manual segmentation and sufficient for our application. We notice that the approach developed has been implemented to be easy and efficient for our TEM images, but it has also been implemented and tested with other tools: by increasing the Gaussian kernel and keeping the window size constant for the multiresolution transform, by performing the gradient transformation with the Prewitt and the Sobel filter...

6. CONCLUSION

The proposed segmentation relies on the identification of the edges at different resolutions, which is well adapted to TEM images of membranes where the contrast is locally enhanced through staining and where the edges have different widths and contrast. It has been shown that a scale-adapted threshold allows an optimal quantity of noise removal and an increasing number of edges are detected. The edges that are contrasted enough are detected with precision at the fine scales, while the others are detected after smoothing at more appropriate coarser scales. The homogeneous areas delimited by the edges on the RGL image are then identified with the watershed method. The resulting segmentation gives acceptable results for many different images acquired with different microscopes, by representing membranes of different sizes and more or less aggregated. It works in many cases, with only a few missing edges. According to the difficulty of segmenting such noisy, low contrasted and different images, the segmentation is considered to be encouraging and useful for the characterization of the specimen and the identification of the ROI.

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7. REFERENCES


