AUTOMATIC METHOD FOR CAVEOLAR STRUCTURE DETECTION AND INTENSITY DISTRIBUTION ANALYSIS FROM MICROSCOPY IMAGES

ABSTRACT

Caveolar structure analysis can be helped with an improved caveolae intensity measurement method and by using a genetic algorith in intensity distribution estimation. Simulations show effectivity and exactness of the new method in corresponce to the conventionally used method, and in addition a higher number of observations can be acquired from an image. Intensity distribution estimation with a genetic algorithm avoids problems like local extreme values which disturb regular curve fitting.

1. INTRODUCTION

Caveolae are plasma membrane pits that are formed upon oligomerization of caveolin proteins i.e. once caveolin proteins assemble into larger complexes [1]. Each caveola is considered to contain a set number of caveolin molecules (Pelkmans and Zerial [2] have estimated this to be 144 ± 39). These complexes can be recognised in the microscopic image (See Figure 1) when protein is fluorescent treated. The number of caveolin proteins in each caveolae complex is the point of interest, and this can be estimated by measuring the intensity of the caveolae in the image.

Caveolae complexes have a tendancy to stack together forming groups of two or more, although single complexes are most common. Theoretically intensities therefore form quantal groups according to the number of caveolar complexes grouped together. Usually fair majority of caveolae structures belong to the first four quantal clusters, and the later groups have too few observations to be statistically reliably analysed.

Images from cells at microscopic level are blurry and noisy containing pixel-to-pixel variation, which must be taken into account. Caveolae appear in images as bright and fairly symmetric circular dots of variable sizes. Detecting caveolaes and separating them from background can be difficult and especially the determination of caveolae intensity automatically is challenging and inexact. Some caveolae are also located so close to each other that they disturb each others intensity estimation.

The commonly used method for measuring intensity of a caveolar structure is described in [2]. Briefly, five rings are formed around the center pixel of a dot with each ring having one pixel longer radius than previous one. First ring contains just the center pixel and the outmost ring has a diameter of nine pixels. Average intensity within each ring is calculated and average value of the outmost ring representing background is subtracted from all values. A one-dimensional normal distribution is then fitted to these values symmetrically set around center pixel value to represent radial sweep, and the area under the curve is used as an estimate of the dot intensity.

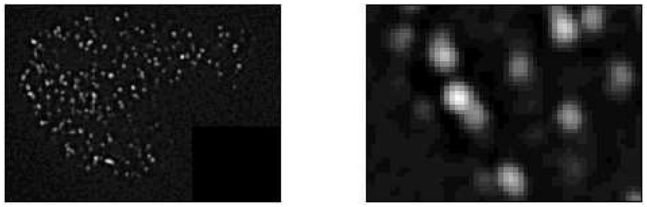

Figure 1: A cell with caveolae and a zoomed detail.

Although this conventional method is suitable for excluding the effect of pixel-to-pixel variation, it lacks seriously of capability to handle closely located dots. This has lead into excluding all the closely related dots from the analysis and therefore reducing the amount of observations, which naturally decreases statistical significance of results and deductions. While the number of observations in a quantal cluster varies from about a hundred to just couple of dozens, it is crucial to obtain as many intensity values as possible. Also systematic filtering of the data (e.g. by the distance to the nearest dot) creates unreliability to statistical deductions.

After intensities have been measured, estimating intensity distribution poses an optimization problem, which regular curve fitting can't solve sufficiently. Fitting a curve is highly dependent on the initial quess and requires therefore manual adjusting and setting. Identifying and separating the quantal clusters and estimating respective curve parameters needs an efficient and exact method, which would increase the quality and reliability of parameters of interest (e.g. proportion of observations in each quantal cluster, widths of clusters). In this study a genetic algorithm is applied for this purpose.

2. THEORY AND METHODS

A single caveolar structure is smaller than the pixel size in a microscopic image, and therefore caveolar structures can be seen as multinormal-like distributed dots in the image. For simplicity, a caveolar structure (single or a group of several) is brutally referred as a 'dot' from now on, and more sophisticated and exact notion is used when needed.

Dots are first recognised simply by finding all the pixels which are brighter than the eight pixels surrounding it. To prevent most of the background pixels to be chosen, the im-

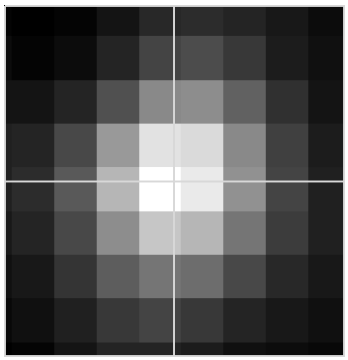

Figure 2: A caveolar structure from a cell divided into quarters according to estimated center point.

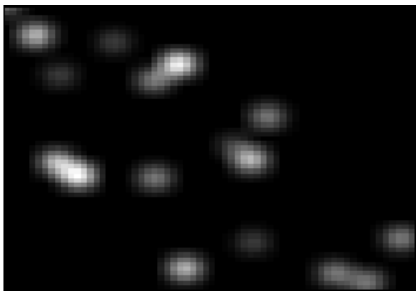

Figure 3: A detail of a simulated image.

age must be smooth out before detection for example by deconvolution. This way all the highest valued pixels of each caveolae should be recognised with a group of background pixels. Background detections are not separated from correct detections (caveolae) at this point, and their intensity values are also calculated the same way as caveolae. If needed however, the lowest value within each nine times nine pixel region could be set as a background value or a common background value from the whole image could be chosen, and subtracted from the mini-image. However, when not comparing absolute intensity values from different images, there is no need for any background correcting at this stage, while each intensity estimation contains presumably equal amount of background.

If the dots were completely separated in the image i.e. far from each other, the intensity of a dot would be easy to measure by just calculating the sum of pixel values within the dot. This would correspond linearly to the amount of fluorescensing proteins in a pit, or a volume of the pit when the intensity is taken as a third dimension. This philosophy is the basis for the method introduced here and the following procedure is performed to measure the intensity of a dot. The exact location (center point) of the dot is needed for this method. Center point is assumed to be inside the brightest pixel of the dot, and its location is estimated by fitting a bivariate normal distribution to nine centermost (three times three) pixels of the dot. It is therefore assumed that the dot intensity distribution is close enough bivariate normal distribution, that the true center location can be estimated by the mean of the best fitting bivariate normal distribution.

Finding the best fit is here done in two parts; first a matrix of possible parameters (mean is restricted inside center pixel, variance below a fixed constant) is tested for goodness of fit, and the best fitting point is chosen as an initial value for the Matlab search algorithm, which finds normal distribution with minimum squared error against the centermost nine pixels of the dot. Using a larger center environment would give us a more reliable fit for the bivariate normal distribution, but is not used because of it is noticed to be too sensitivity to the neighboring dot disturbance producing highly erroneous results. Majority of dots in true images are enough separated, that the centermost nine pixels stay undistrubed.

According to the estimated true center the image is divided into four square-shaped quarters of size four times four pixels and the pixel values falling inside the four squares are calculated. (See Figure 2.) Values from pixels partially inside squares are of course summed only partially according to their area inside the square. Quarter values are multiplied by four to get an estimate for the whole dot intensity. Most of the dots in images are small enough that they fit into this eight times eight pixel area. If there are other dots nearby, the highest quarter value probably contains the highest amount of disturbance and the lowest value of these quarters would presumably to be the least disturbed. However, tests have shown that the second and third highest valued quarters are the most reliable estimators for the whole dot intensity. This is probably because the highest and lowest quarters are also most sensible to error produced by false estimate for the center location of the dot. Therefore, either second or third largest quarter value should be used as intensity estimate.

After intensities have been measured, the value distribution must be truncated to exclude background values from the low end and values belonging to highest clusters from the high end. Clusters that have so few observations that they have no statistical significance, should be removed, while they disturb the cluster parameter estimation algorithm. Usually these truncation points should be visible as very low frequencies (bars) between higher frequencies in the histogram, and especially the first quantal cluster should be evident if the measurement has been succesful and correct (see also Figure 5. If location of each detected dot in the image is stored during intensity estimation process, a caveolae of an assumed quantal cluster can be found in the image and assumption confirmed or corrected.

Intensity distribution is assumed to be a finite mixture of several one-dimensional normal distributions, each normal distribution representing of a quantal cluster with variable number of caveolae and with some estimation error (see [3] for finite mixture models). Fitting a mixture model to the intensity distribution establishes a complex optimization problem, because no specific assumptions of distribution parameters (mean, variance and mixture parameter for each normal distribution) can be made. This can be solved efficiently and exactly with a genetic algorithm [4], developed initally for brain imaging applications. Genetic algorithm maximises the likelihood function with respect to the parameter vector.

3. DATA AND RESULTS

3.1 SIMULATION

Simulated images are created to test the accuracy of intensity measurement in certain conditions. Simulation with an image consisting of only constant valued unfocused dots without any disturbance from neighboring dots is used to determine the distribution created by inaccurateness of a measure-

	True value	Mean	Standard deviation
Old method	10 029	9 754,3	170,6
New method	9 830	9 798,1	24.8

Table 1: Measurement accuracy with undisturbed single dots.

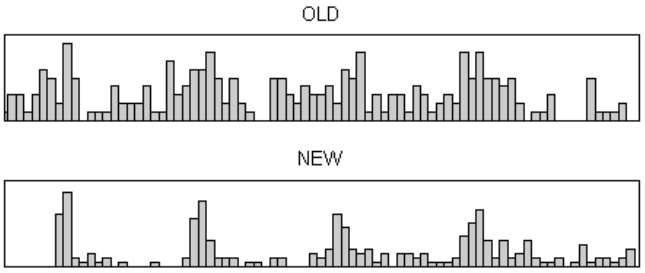

Figure 4: Results from simulation image with multiple dots.

ment method itself. Another test is done with an image with multiple dots to test the capability to measure intensities from closely located dots. While the true intensity values in simulated dots is known, correctness and error rates from measurement can be observed.

A simulated dot was sized nine times nine pixels and created from a bivariate normal distribution, whose mean was located randomly inside the center pixel to simulate out-of-focus effect. Dot pixel values are cumulative distribution values of multinormal distribution. The dot was finally multiplied with a constant to achieve total intensity. A bigger simulation image consisting of multiple dots were created by adding mini-images to the 200 times 200 pixel image, locating each dot randomly allowing partial and complete overlapping.

Results of the test with 1000 single unfocused dots without any noise caused by neighboring dots (each mini-image was estimated separately) can be seen in the Table 1. True value column is measured from a 'perfect dot', i.e. one centered exactly at the center pixel and distributed completely symmetrically without error or distrubance. While the measurement methods are different, the true values which to compare are also different (old method uses background correcting, new doesn't). As can be seen, new method performs significantly better with this simplified simulation having mean value much closer to the true value and substantially lower deviation. Of course, true images and dots are not this perfectly distributed, but it would be a base requirement for a measurement method to produce good results at least in optimal conditions. In studies concerning caveolar structures it is usually sufficient to have measurements which can be separated to quantal clusters. These variations are more than enough accurate for that purpose, but the disturbance from neighboring dots is not included yet.

While the purpose in previous simulation was to test capability to measure invidual dot intensities, in the bigger simulation image the purpose is to test the ability to measure tightly stacked dots and clusters. There are four different constant intensity values for dot intensities to represent quantal clusters, so that theoretically intensities should form a histogram with four equally high bars located at constant distances apart from each other. Because dots are located randomly throughout the image and some of them are overlapping each others forming extra bright dots, higher quantal groups can have higher frequency than expected (e.g. there can be a $4\times$ constant dot which actually consists of overlapping $1\times$ and $3\times$ constant dots.) Also fifth quantal groups was formed in the image which was not initially intentionally created.

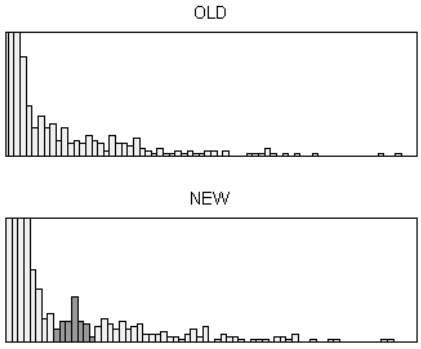

Figure 5: Intensity histograms of measurements from a real cell.

Intensity measurement results from big image can be seen in Figure 4. Numbers of created dots in the image is 400, of which 265 were detected. Therefore significant ovelapping and neighbor disturbance is present. Based on the figure new method seems to have a better capability to estimate intensities of stacked dots. The quantal clusters are clearly more recognisable in the new method results and while there seems to be less overlapping amoung cluster distributions. The difference in variation of dot intensity estimations between the method is also visible.

3.2 MICROSCOPY DATA

A real cell image is acquired with a confocal microscope and deconvolded to reduce pixel-to-pixel noise. Cell is in normal conditions, so there is some prior knowledge based on earlier studies about what sort of intensity distribution is to be expected. Dots are detected as earlier, and intensities of all detected dots, including background, are estimated with both methods.

While the true distribution behind estimates is not exactly known, evaluation of the quality of results is more difficult. Histogram of the results can be seen in Figure 5. The width of histogram frequency bars is set to be visually informative and to show the overall intensity distribution. With more carefult interval setting and adjusting quantal clusters would be more visible in both methods. Observations assumed to belong to the first quantal cluster in the new method results are in dark colored bars. Note that the first quantal cluster is clearly distinguishable in the new method histogram, but the later clusters are more unclear. But for exmaple using Parzen window might reveal also other quantal clusters.

3.3 DISTRIBUTION ESTIMATE

To demonstrate the performace of genetic algorithm in estimating distribution of intensities, another real cell intensities are estimated with the new method and processed (truncated, image scaled) to be visual. Caveolae from this cell have an intensity distribution which is more easily recognised than in previous case. Genetic algorithm is then used to fit a mixture

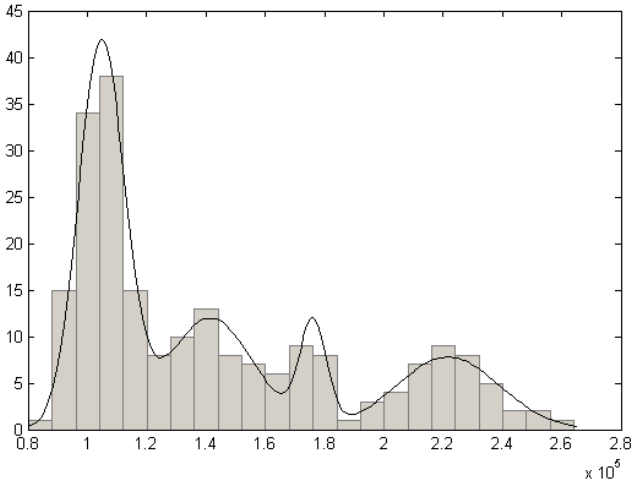

Figure 6: Intensity histogram with fitted mixture model.

model of four normal distributions, representing the first four quantal clusters. The estimated curve is represented in Figure 6 with histogram. Actually, the values used in fitting are from a much more dense histogram, but for visuality fairly large histogram bar width is chosen in figure.

It looks like the algorithm is able to find one of the most probable distributions to the data. The goodness of fitting results can usually be seen by eye while only a small number of quantal clusters are estimated, and in this case fit seems to be quite exact. Distribution parameters can be acquired from the algorithm and further analysis proceeded according to them. However, not in all cases the intensity data is so clear and easy to fit as in this illustration and some considering might be needed with the results. Also it should be remembered that genetic algorithm is a stochastic method, producing slightly different results every time. Therefore monitoring the goodness of results is in order.

4. DISCUSSION

The method introduced here clearly performs better in simulations with both separated undisturbed dots and with tightly stacked disturbed dots. The difference in performance is based on the improved capability to measure unfocused dots and excluding the effect of neighboring dot disturbance. Solving the second problem (close locations), increases the number of observable caveolae and reliability of statistical deduction.

Naturally, results with true caveolae are not so straightforward to compare but because simulation images were made to be as similar to the true images as possible, it is highly probable that this new method produces more exact measurements. While the performance difference is so high in simulations, diverse results are to be expected also from true caveolae images. However, the judgement which method produces more correct results, is still to be done.

Genetic algorithm works well in finding caveolae intensity distribution parameters and best of all it is done automatically. Choosing a suitable data formation for the algorithm shouldn't pose a problem, while algorithm performs well with quite a wide range of them. Histograms or Parzen windowed data can be used for example, with relatively short interval values producing detailed data. Clearly the most vulnerable spot in this new method is the approximation of true location of the dot. Poor estimates in this can cause radical changes in the intensity quarter values. Although using the second and third largest quarter values slightly eliminates this problem, it still would be an improvement to avoid the need for centering. Bivariate distribution fitting algorithm used here is fotunately one of the simplest methods that can be used, so there is a lot of room for improvement. Better method for centering would improve especially the quality of measurements from real caveolae which might not fit so perfectly to normal distribution.

If image is considered as a plane or a surface and the intensity values as a third dimension orthogonal to the plane, then the dots would be as multinormal distributed hills on the surface. A perfect method, i.e. a method which would extract all the information from the image, would then be the one which estimates all individual multinormal distributions straight from the image. Then individual variances, volume below each hill and other parameters could be measured within the limits of some restrictions and each caevolae would get an individual set of parameters. This method however would require an algorithm capable of estimating overlapping multinormal distribution parameter reliably and efficiently, which is problematic with current algorithms. Suitability of multidimensional version of genetic algorithm will be tested though.

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