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Applying Pattern Recognition to High-Resolution Images to Determine Cellular Signaling Status

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Abstract

Two frequently used tools to acquire high-resolution images of cells are scanning electron microscopy (SEM) and atomic force microscopy (AFM). The former provides a nanometer resolution view of cellular features rapidly and with high throughput, while the latter enables visualizing hydrated and living cells. In current practice, these images are viewed by eye to determine cellular status, e.g. activated versus resting. Automatic and quantitative data analysis is lacking. This work develops an algorithm of pattern recognition that works very effectively for AFM and SEM images. Using rat basophilic leukemia (RBL) cells, our approach creates a support vector machine (SVM) to automatically classify resting and activated cells. 10-fold cross-validation with cells that are known to be activated or resting gives a good estimate of the generalized classification results. The pattern recognition of AFM images achieves 100% accuracy, while SEM reaches 95.4% for our images as well as images published in prior literature. This outcome suggests that our methodology could become an important and frequently used tool for researchers utilizing AFM and SEM for structural characterization as well as determining cellular signaling status and function.

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I. Introduction

Cellular shape, morphology, and intracellular membrane features are frequently used as direct readouts of the biological status of the cell, such as activation, spreading, motility, malignancy and death [1–10]. Well-known examples include donut-shaped versus sickle-shaped red blood cells indicating a healthy and ill status [11, 12], dendritic versus relatively smooth morphology are associated with mature and immature dendritic cells (DCs) [13–16]. Modern bioimaging technology enables visualization of cells with much higher spatial resolution than conventional optical microscopy. Scanning electron microscopy (SEM) allows the shape as well as detailed cellular features such as filopodia, lamellar podia, podosome, and microvilli to be visualized [17–22]. While simple and rapid, SEM requires cells to be dry and stained with heavy metal ions to enhance contrast [17–22]. Atomic force microscopy (AFM), although relatively low in throughput, enables visualizing cellular morphology and structural features for hydrated and even living cells in near-physiological conditions [23–31]. Researchers have just begun to explore the qualitative and quantitative information from these high resolution structural features in correlation with the biological status function of cells, such as the degree of motility and malignancy [32–37].

Using a specific cell type as an example, rat basophilic leukemia (RBL) cells, prior investigations by us and other researchers have studied the cellular activation processes, in the investigations of allergy [4, 15, 29, 30, 38–47]. These investigations revealed that these nanometer- to micrometer-scale structural features are directly related to cellular signaling processes [4, 15, 29, 38–45]. In the resting state, the RBL membrane is relatively smooth as characterized by the presence of microvilli, shown in Fig. 1a and Fig. 1c. Upon IgE antibody-mediated activation, also known as immediate-type hypersensitivity response or allergy, folded features such as ridges are observed on cell membranes, illustrated in Fig. 1b and Fig. 1d [28–30, 43, 47–49].

At present, these high-resolution images are viewed by eye as a qualitative readout for the activation status of the RBL cells [20, 26, 28, 43, 47]. Systematic, automatic, and quantitative data analysis is severely limited [45, 50, 51]. The challenges include the complexity of cellular systems, the relatively small number of high resolution images in comparison to material imaging, and the detailed communication and collaboration required among scientists in imaging, cellular bioscience, and pattern recognition. Our past work has demonstrated the importance and effectiveness of utilizing machine learning for experimental data analysis [52–54]. This work reports our approach which introduces pattern recognition as part of a data analysis of these high-resolution images of RBL cells, and uses the outcome to classify each cell based on activation status. Among various tools and approaches utilized in pattern recognition, we have identified and developed approaches that best address the challenges of this work. More detailed discussion of experimental design and validation can be found in supplementary materials. For this work, 35 AFM images were acquired from 8 independent sets of experiments. Among them 10 RBL cells were in a resting state, and 25 were activated via antibody mediated processes. In addition, 99 SEM images were taken from 13 sets of experiments. Among them, 43 RBL cells were resting, and 56 were activated. The optimization and simplification are performed to enhance speed and efficiency of our pattern recognition algorithms. The results exhibit very high

accuracy in determining the activation status of RBLs. We anticipate this approach shall have generic applications to a wide range of cell types and signaling processes. Further development shall harness important biological insights from the high-resolution images that modern technologies enable.

II. Image Preprocessing

AFM and SEM images of RBL cells are typically acquired by optimizing experimental conditions to reveal cellular features that are of interest to individual researchers. The technical specifications and user skills vary from one laboratory to another. As such, the image size, signal-to-noise ratio, background, and contrast often vary and are highly nonuniform and non-standard from image to image and from one laboratory to another. Therefore, preprocessing is necessary to assure that structural features pertaining to cellular signaling processes are revealed accurately before applying pattern recognition. In this work, each cell undergoes a process of background subtraction, noise reduction, and finally circular cropping to a standard 512×512 pixel resolution, as summarized in Fig. 2. These steps assure localizing the cells while normalizing the size. These standardized circular images are ready for the subsequent steps in pattern recognition (see Section III).

The image processing steps are identical regardless of the nature of images, e.g. AFM or SEM. Fig. 2 reveals the key steps and corresponding results for images acquired using AFM (left) and SEM (right), respectively, to illustrate and compare. The first step in preprocessing is to enhance intrinsic cellular features in the images by eliminating the background present in images. Since the background is highly local, we adopted a white top-hat transform (WTHT) [55]. The WTHT is designed to extract each feature (peak in imaging contrast) in an image by subtracting the background surrounding it. The WTHT is defined as the difference between the input image and its opening using

$$T_w(I) = I - I \circ S \quad (1)$$

where I is the input image, $T_w(I)$ is the white top-hat transform, S is a structuring element and $\circ$ is the opening operator. An opening with a 10×10 circular structuring element was used. The gray-scale opening serves to filter out the cellular features, leaving only the general background patterns. The image formed after the opening was then subtracted from the original so that only the features remain.

After performing the WTHT, the result was normalized so that the darkest pixel is 0 and the brightest is 1. A 7×7 median filter was then applied to remove any noise and only allow true features. Next, an adaptive threshold was applied to convert the grayscale image to black and white. The adaptive threshold finds the appropriate threshold for each region of an image. The image was severely blurred with a 25×25 average filter and the difference between the filtered image and the original served to further increase contrast. Finally, a standard gray threshold was applied using Otsu's method [56] to determine the threshold.

For RBLs under this investigation, we added one more step to enable cell selection even in the case of congregation or confluence of cells causing overlap at cellular peripheral regions. Considering the focus of this pattern recognition targets membrane features, we subjected the black-and-white images to circular cropping. A morphological closing operation with a 9×9 disk structuring element was performed to fill in small holes in the resulting black-and-white images. Then the largest connected component of white pixels was taken to be the cell, from which the centroid, (x, y) and radius, R , were calculated. Then, a square mask containing the circle with center (x, y) and radius of $0.9R$ was applied to original image. Only the pixels within the circle were kept and the rest were set to 0 (black) so that only membrane features were analyzed. The center and radius of the cell are used to crop the original image so that it contains only the cell image. The cropped images were then scaled to a square of 512×512 pixels so that the feature generation step has uniformly-sized input images. The resulting image representing the initial cellular data was utilized for the next step of feature generation.

III. Feature Generation

The goal of the feature generation step is to create a set of data that can be used by the classifier to distinguish activated from resting cells. Since the cell's biological status is correlated with overall cellular shape, geometry, size as well as membrane features [28–30, 38, 47–49], our feature generation and selection must reflect upon these structural characteristics. Two feature generation methods that we decided to employ for recognizing nanoscopic features of RBL cells are Local Binary Patterns (LBP) and Histogram of Oriented Gradients (HOG). LBP [57] was designed to describe textures in an image. Since the difference between activated and resting RBL cells lies primarily in the membrane features, density, and size of villi and ridges, texture differences are a dominant feature. HOG [58] has been proven to be effective for recognizing object shape and geometry; for example, it is often used for differentiating humans from other objects in images [58–60]. In order to determine activation status of mast cells and, at the same time create a more general system capable of accessing other cell signaling processes for a wide range of different cell types, combined LBP and HOG features are necessary to achieve accurately classification.

A. Local Binary Patterns

We applied LBP by calculating a binary code for each pixel location from the grayscale image. The code was created by comparing the center pixel in a 3×3 area and its neighbors, as shown in Fig. 3. If the center pixel was greater in value than its neighbor, the number 0 was written in its place, otherwise the pixel has the value of 1. Starting with the top-left value and moving clockwise, the 8-bit binary value was created from the 0's and 1's. Therefore, for each pixel in the image, a new number between 0 and 255 in decimal is generated. This process is illustrated in Fig. 3.

The image was then divided into 256 windows of size 32×32 . In each window a histogram was created of the 1024 binary codes encountered. A window size of 32×32 was chosen to match the scale appropriate with the ridge features in the cell images, the vast majority of ridges occupied less than one-third of a 32×32 window.

Using the original LBP method, there would be 256 bins in each histogram. In our algorithm however, uniform binary patterns were used. An 8-bit binary pattern is uniform if it has at most two 0-1 or 1-0 transitions. The example given in Fig. 3 is non-uniform because it has four transitions. The histogram is made by creating a bin for each uniform pattern and placing the sum of all non-uniform patterns in a single bin. There are 58 uniform patterns, so there are 59 total bins.

Each histogram is then normalized using the L^2 (Euclidean) norm, so that the new histogram values are given by

$$h_n = \frac{h}{\|h\|_2} = \frac{h}{\sqrt{h_1^2 + \dots + h_n^2}} \quad (2)$$

where h is the histogram vector and h_n is the normalized histogram vector. The resulting feature vector is formed by concatenating the normalized histograms. Our 512×512 images were divided into 32×32 windows, resulting in $16^2 = 256$ histograms. Since each histogram has 59 bins the feature vector was 15,104 elements long.

B. Histogram of Oriented Gradients

We found that adding HOG feature descriptors to LBP more completely represented cell status. The HOG starts by computing the gradient of the image, given by

$$\nabla f \equiv \begin{bmatrix} G_x \\ G_y \end{bmatrix} = \begin{bmatrix} \frac{\partial f}{\partial x} \\ \frac{\partial f}{\partial y} \end{bmatrix} \quad (3)$$

where $\frac{\partial f}{\partial x}$ is the gradient in the x-direction and $\frac{\partial f}{\partial y}$ is the gradient in the y-direction [61]. Thus from the input image, two output images are generated: the G_x and G_y gradient images. By taking

$$\frac{\partial f}{\partial x} = f(x+1) - f(x-1) \quad (4)$$

for $\frac{\partial f}{\partial x}$ and similarly for $\frac{\partial f}{\partial y}$, we can produce the symmetric 1-dimensional filter $[-1 \ 0 \ 1]$ and its transpose $[-1 \ 0 \ 1]^T$. Thus we compute G_x by

$$G_x = [-1 \ 0 \ 1] * I \quad (5)$$

and G_y by

$$G_y = [-1 \ 0 \ 1]^T * I \quad (6)$$

where I is the input image and $*$ is the convolution operator. Rather than the gradient in the x - and y -direction, the HOG method utilizes the magnitude and direction of the gradient given by

$$G = \sqrt{G_x^2 + G_y^2} \quad (7)$$

$$\theta = \tan^{-1} \left(\frac{G_y}{G_x} \right). \quad (8)$$

The HOG method used subdivides the image into 8×8 windows. Just as with LBP, HOG creates a histogram over each window. Except with HOG the histogram bins are based on the orientation of the gradient at each pixel and therefore a window size smaller than 32×32 , (i.e. one quarter of the size) is desirable to concentrate on more detailed pattern orientation. Here the absolute value of the orientation angles were taken and placed in 9 bins evenly spaced between 0 and 180° . This partitions the oriented gradients into 20-degree increments which is representative of the relatively smooth and few directional changes of the structural features observed in activated cells. The histogram was created by adding the sum of the magnitudes of each gradient that are within a bin's range of angles. For example, the magnitudes of all gradients in a single 8×8 window with angles in the range $[0, 20)$ are summed to form a single bin.

The histograms for each window were then normalized. To account for changes in contrast throughout the image, normalization was performed within blocks surrounding each window. The block size used in this method is 2×2 , again one quarter of the size of the HOG window, with an overlap of one window width. An example is shown in Fig. 4. The example image is made up of 6 windows W_{ij} for $i=1,2$ and $j=1,2,3$. The histograms of each window W_{ij} is $H(W_{ij})$. To create the first block the windows $H(W_{11})$, $H(W_{12})$, $H(W_{21})$, and $H(W_{22})$ are concatenated. Since the overlap is one window wide on each side, block 2 is created by concatenating $H(W_{12})$, $H(W_{13})$, $H(W_{22})$, and $H(W_{23})$. After each block is created it is normalized in the same way the LBP histogram vectors are normalized, using equation 2. Concatenating all the normalized blocks gives a feature vector for the entire image that is 142,884 elements long.

IV. Feature Selection and Classification

A. Feature Selection and Cross Validation

Feature generation methods such as LBP and HOG produce very large feature vectors. Using all of these features for classification is neither time efficient nor necessary as not all

features are biologically “equal” in the context of cellular biology and imaging contrast. Many of the feature vectors are redundant, unnecessary, or insignificant. Therefore, extracting meaningful features from the whole set is a necessary and important step for fast, accurate classification. A Welch’s t-test [62] was employed which exploits the use of a null hypotheses to understand the variation among the vast number of features in predicting the known classes. The Welch’s t-test has been used successfully in prior investigations to select features that contribute most to classification, proven particularly effective for applications with a limited number of training data where each class does not necessarily have equal variance among samples [63, 64]. Welch’s t-test assigns a p-value to each feature and it is used to determine how useful the feature is at discriminating between the two classes. A Welch’s t-test was used over a standard t-test due to the features and classes having unequal variances. This type of ranking with p-values can be used to reduce the amount of features used.

Automatic recognition of activation status is accomplished through a classification process. In pattern recognition, a set of features is mapped to a label. Classification is a type of pattern recognition that labels the input set of features with one of several classes. In this case the classes are the two RBL cell states of interest: resting and activated.

Many methods for classifying input feature vectors exist. We chose to use a support vector machine (SVM) since we have a smaller number of data points than features. This method uses hyperplanes to divide the two classes of interest, in this case an activated or resting RBL cell. Each feature point is comprised of a large number of individual feature values provided by the histograms created using LBP and HOG as selected during feature reduction. A linear kernel modeling the hyperplane was used and solved by the sequential minimal optimization algorithm [65]. Ultimately, one hyperplane is chosen which either maximizes the distance between the plane and the feature points of each class, or minimizes the amount of misclassified feature points on either side of the hyperplane, depending on whether or not the data are linearly separable [66].

We also tested the performance with another known pattern recognition protocol, a decision tree approach [67]. Systematic comparison reveals that SVM finds a more optimal partitioning of the feature space than a decision tree and ultimately yielding more accurate assignments of cellular signaling status. On the other hand, the decision tree approach is faster and more effective if training data sets are large. For determining RBL cell activation, a large number of training data are not necessary to achieve accurate results. Therefore SVM is more desirable.

To ensure the validity of the SVM classifier on more than just a single training set of images, 10-fold cross-validation was utilized. Cross-validation has proven valid and effective in situations where the number of images or data is less than massive [66, 68–70]. We note that 35 AFM images of RBL cells may seem to be a limited data set, but this number is among the highest in peer investigations [71–73]. SEM technology is relatively higher throughput, and 99 images were used in this work, with more images available in literature. In the interest of developing a generic algorithm, the same cross-validation was applied to SEM images.

In our cross-validation method, the full set of images available was partitioned into 10 disjoint subsets randomly. In each fold, a single subset of the 10 was kept aside for testing the model and the remaining 9 subsets were used as training data. The SVM classifier was trained on the 9 training subsets and the results of the fold are recorded. This process was repeated until all 10 subsets have been used for testing. The correctly classified and misclassified results of each fold were tallied to produce the accuracy measure of the 10-fold cross-validation.

Due to the random assignment of subsets in the cross-validation, the results can vary slightly between trials. To reduce the variance of the cross-validation results 30 independent trials are performed. In each trial, 10-fold cross-validation is performed with a new randomly-divided set of subsets. The 30 results are averaged to create the final accuracy for the classification method.

B. Classification Using Various Types of Images

SEM images typically consist of a single type of contrast, the intensity of secondary electrons at the detector, while the AFM images are acquired from multiple contrast mechanisms: topography, deflection, and lateral force images. In each contrast type, our algorithm iteratively generates a profile of classification results using the feature selection method described. Fig. 5 shows the results of classification given the top 1 to 40 features selected by the p-values. For each feature number, 30 trials were run to reduce variance of the results. The trend and detailed feature number dependence guide our choice of image type and the minimum feature numbers needed for accurate pattern recognition in our chosen investigations such as AFM or SEM. As shown in Fig. 5, the feature number dependence varies with the origin of the contrast. Topography which measures the physical height of surface structures, yields a high accuracy of 100%, at 10 features. Deflection which measures AFM cantilever bending when scanning over surface structures has similar behavior. Lateral force images result in slower increases and do not reach 100% accuracy until 29 features are used. To determine the number of features for the algorithm, we chose the number of features in which there is less than 1% change in accuracy upon increasing the number of features by one with such stability maintained in 10 consecutive increases. For AFM topography, the transition occurs at 32 features, that is, little changes are seen from 33 – 43. For deflection and lateral force, transitions are 20 and 14 features, respectively. This approach is not limited to these three types and could include others such as elasticity, amplitude, and phase in AFM imaging. Based on the trend and stability, we decided to use AFM topographic images at 40 features. For SEM images, the stability is not reached until the number of features is much higher as shown in Fig. 6, where 95.7% accuracy is reached and stabilized at 135 features. Therefore, at least 135 features are needed in our pattern recognition algorithm in the case of SEM image analysis.

These results are very encouraging as the pattern recognition program has no knowledge nor input to reveal physical origin of image contrast, but seems to yield more accurate results using fewer features from image types with a clear physical base, such as AFM topography. As a comparison, the SEM images were analyzed and shown in Fig. 5, where the accuracy increases slower than AFM, and seems to plateau at 90% accuracy upon using 20 features.

The secondary electron intensity is not as simple as AFM topography because many factors could contribute to the contrast such as topography, OsO₄ distribution, and potential distribution due to material and physical features [17, 74–76]. To test how many features are required for accurate SEM classification results, cross-validation and accuracy measurement was performed for an increasing number of features from 1 to 160 in order of p-value computed by the Welch's t-test. While the Welch's t-test ranks an individual feature's ability to discriminate between classes, the accuracy does not necessarily increase monotonically with increasing numbers of features. It is more meaningful to observe the trend, as shown in Fig. 6. The graph of the accuracy measured for the SEM data is given in Fig. 6 with the AFM topography image accuracy shown for comparison. In these cases, more features, up to 135, seem to provide an effective remedy to ensure accuracy.

V. Results And Discussion

To reveal and evaluate the performance of our method, a set of AFM topography images of RBL cells were first used which contains 10 resting and 25 activated RBL cells for a total of 35 images. Following the image preprocessing and normalization, the 35 images were divided into 10 subsets, the first 5 contains 3 images/subset while the other 5 have 4 images/subset. An SVM classifier is created and its performance is measured, following the protocols discussed previously.

The results of the AFM image classification are shown in Table I. Based on the protocol from the previous section, 40 features were selected and used for recognition. The outcome is reported in a confusion matrix. Confusion matrices reveal the correctly classified results as well as the false positives and negatives. Since the training and testing sets for cross-validation were chosen randomly, 100 independent trials were run. The results shown are the averages of those trials with the standard deviation. The accuracy is calculated by taking the sum of the diagonal of the confusion matrix which represents the number of correct classifications, divided by the total number of cell images. In the case of AFM image classification, all 25 activated cells were classified correctly by our algorithm and all 10 resting cells were correctly identified. Therefore, 100% accuracy is achieved. To compare to SEM results later, we used 135 features, yielding the same accuracy of 100%.

The experiments were repeated using the same method with 99 SEM images. If 40 features were used, the accuracy only reached 90.1%, as shown in Table II. On average 38.9 of the 43 resting cells and 50.3 of the 56 activated cells were correctly classified which gives an overall accuracy of 90.1%. The 95.4% average accuracy can be achieved when increasing the number of features to 135, which yields optimal results with our method using SEM images.

To test the performance of our methodology, we randomly and blindly selected six SEM images that were acquired and published by other teams [47, 77]: three resting RBL cells and three more activated. These images were cropped from the paper's PDF file. Using the images taken directly from the papers, our classifier rapidly and correctly identified the cell status for 5 out of the 6 images using only 135 features. Upon closer examination of the only

false classification, our program indicates a low signal-to-noise ratio associated with that particular image, which was cropped from the publication in pdf format.

To test the robustness and limitations of our approach, we repeated our experiments using 2-fold cross-validation, that is, 50% of the images were used for training and the other 50% were used for testing. As before, the images were split into the training and testing sets at random, 100 times, to reduce variance. For 40 features, the average accuracies of this validation method were 90.9% and 99.5% for SEM and AFM topography, respectively. For 135 features, the average accuracies were 95.1% and 99.5% for SEM and AFM topography, respectively. We infer that our 10-fold cross-validation is sufficient to ensure accuracy in terms of determining cellular activation.

VI. Conclusions

This work represents our initial attempt to provide an accurate and automated system for determining cell signaling status from high resolution images of single cells. Both AFM and SEM images were utilized to test our approach. Procedures included using local binary patterns and histogram of gradients as features, reducing the feature space using t-test analysis, and training a support vector machine. Using this procedure we demonstrated that our system is capable of finding the most useful contrast type for determining cell activation status accurately. More than 95% accuracy was achieved for AFM and SEM images. For situations with characteristic structural features such as membrane ridges captured by AFM, 100% accuracy was attained with only 10 features. Even with mixed contributions to image contrast, such as SEM images, more than 95% accuracy was obtained with 135 features. These initial successes are very encouraging. Work is in progress to investigate more cell types and various signaling processes to further optimize these data analysis algorithms. We selected mast cell activation as a starting point to demonstrate the complexity and effectiveness of applying machine learning and pattern recognition to cellular biochemistry research. Note that mast cell activation is a relatively simple problem in comparison to the complexity of cellular images. We fully anticipate that more pattern recognition tools and protocols will have to be added and developed when facing more complex cellular imageries. With more systems and investigations, we envision a similar machine learning method shall become a generic image analysis tool to benefit cellular-based bioscience and biomedical research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Characteristic AFM images of (a) a resting RBL cell, and (b) an activated RBL cell. Characteristic SEM images of (c) a resting RBL cell, and (d) an activated RBL cell. Scale bar equals 5 μm .

Fig. 2.

Key steps in our data processing (middle) and the outcome in each step using an example AFM image (left) and an SEM image (right).

Fig. 3.

An example illustrating LBP: a section of grayscale image to binary code.

Fig. 4.
Example section of grayscale image to binary code blocks.

Fig. 5.

Accuracy of activation classification from AFM images as a function of the number of features used. AFM image types include topography, deflection and lateral force contrast, respectively. Results from SEM images are also included for comparison. In each data point, the standard deviation was within $\pm 3\%$.

Fig. 6.

Accuracy of both SEM and AFM classification versus the number of features used. For all experiments with greater than 10 features, the standard deviation was within $\pm 1.5\%$. Experiments with between 1 and 10 features had standard deviation within $\pm 3\%$.

TABLE I

Classification Results From AFM Topography Images

	40 Features		135 Features	
	True Resting	True Activated	True Resting	True Activated
Predicted Resting	10 $\pm$ 0	0 $\pm$ 0	10 $\pm$ 0	0 $\pm$ 0
Predicted Activated	0 $\pm$ 0	25 $\pm$ 0	0 $\pm$ 0	25 $\pm$ 0
Average Accuracy (%)	100		100	

TABLE II

Classification Results From SEM Images

	40 Features		135 Features	
	True Resting	True Activated	True Resting	True Activated
Predicted Resting	38.9 ± 0.9	5.7 ± 0.5	41 ± 0	2.6 ± 0.6
Predicted Activated	4.1 ± 0.9	50.3 ± 0.5	2 ± 0	53.4 ± 0.6
Average Accuracy (%)	90.1		95.4	