Automated analysis of protein subcellular location in time series images

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ABSTRACT

Motivation: Image analysis, machine learning and statistical modeling have become well established for the automatic recognition and comparison of the subcellular locations of proteins in microscope images. By using a comprehensive set of features describing static images, major subcellular patterns can be distinguished with near perfect accuracy. We now extend this work to time series images, which contain both spatial and temporal information. The goal is to use temporal features to improve recognition of protein patterns that are not fully distinguishable by their static features alone.

Results: We have adopted and designed five sets of features for capturing temporal behavior in 2D time series images, based on object tracking, temporal texture, normal flow, Fourier transforms and autoregression. Classification accuracy on an image collection for 12 fluorescently tagged proteins was increased when temporal features were used in addition to static features. Temporal texture, normal flow and Fourier transform features were most effective at increasing classification accuracy. We therefore extended these three feature sets to 3D time series images, but observed no significant improvement over results for 2D images. The methods for 2D and 3D temporal pattern analysis do not require segmentation of images into single cell regions, and are suitable for automated high-throughput microscopy applications.

Availability: Images, source code and results will be available upon publication at http://murphylab.wab.cmu.edu/software

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1 INTRODUCTION

Subcellular distribution is an important characteristic of a protein because location is intimately related to its function. The most common method used to find a protein’s subcellular location is to fluorescently tag the protein, take images by microscopy and then visually analyze the images. Previous work has shown that automatic image analysis can outperform visual examination, providing higher sensitivity to subtle differences (Murphy et al., 2003). Proteins from major subcellular locations can be differentiated from each other with over 90% accuracy (Huang and Murphy, 2004). However, previous work has been done using static images and the challenge now is to extend these approaches to time series images that better reflect protein behavior in living cells. One objective is to distinguish proteins that have similar static but different temporal patterns. This is of obvious biological importance since many proteins change their location over time in order to carry out their functions. For example, helicases localize to the nucleus during the G1 phase of the cell cycle to repair endogenous DNA damage, and exit the nucleus during S phase (Gu, 2004).

For automated analysis of protein subcellular locations, machine learning tools for classification and clustering are well established (Glory and Murphy, 2007). A key component is feature design: good features are numerical representations of the patterns that capture the differences between classes or clusters. The goal for temporal pattern analysis is therefore to design good features from time series images that distinguish different protein movements.

Automated analysis of biological time series images has received significant attention in recent years, but most movies are of low resolution at the organ or cell level (Liebling et al., 2006; Souvenir et al., 2008), and only a few deal with high resolution microscopy images that record protein movement within a single cell (Damas et al., 2006; Sigal et al., 2006; Zhou et al., 2009). We have carefully studied applicable theories and algorithms from the computer vision field and adopted/designed five sets of temporal features for application to subcellular pattern analysis for microscopy images.

The first, and perhaps most straightforward, approach to characterizing temporal behavior is to track individual objects in a cell and calculate features to describe these tracks. For example, speed, the most common numerical description of movement, requires measuring the total distance the object travels along its way. However, some proteins do not form rigid objects and it is therefore unclear what the targets for tracking should be. The best example is cytoplasmic proteins: even when they show changing fluorescence distributions, there is typically no easily characterized movement of objects. Such ‘complex and nonrigid’ motion that has statistical regularity was defined as ‘temporal texture’ (Nelson and Polana, 1992). In that work, the normal flow on each pixel was used
to represent movement along the image gradient and compared to uniform direction flow. The concept of ‘temporal texture’ has also been applied to adapt Haralick texture features for static images to movies (Bouthemy and Fablet, 1998) or to develop a series of features based on temporal slices (Ngo et al., 2002). We therefore chose co-occurrence based temporal texture features (Hu et al., 2006) and normal flow features as our second and third temporal feature sets. To design other temporal features without defining an entity for tracking, we have also explored image intensity changes in the frequency domain (Fourier transform features) and analyzed static feature changes over time (autoregression features).

We evaluated the ability of these feature types to improve our ability to discriminate protein patterns in a collection of 4D images (three spatial dimensions over time) for 12 cell lines expressing fluorescently tagged proteins.

2 METHODS

2.1 Image acquisition

NIH 3T3 cell lines previously generated as part of a proteome-scale tagging project were used in this study (Chen et al., 2003; Garcia Osuna et al., 2007; Jarvik et al., 2002). In each cell line, a particular protein is tagged by CD tagging (Jarvik et al., 1996) which introduces a green fluorescent protein (GFP) tag into the gene coding for the protein using self-inactivating retroviral vectors.

Cells were plated on glass-bottomed culture dishes in Dulbecco’s modified Eagle’s media. After 48 h, the media were changed to Opti-MEM to avoid the interference by phenol red and provide pH stability. Three-dimensional movies of GFP tagged proteins in NIH 3T3 cells were taken by a spinning disk confocal microscope. The imaging system consists of a LaserPhysics Reliant 100W 488 Argon laser, a Yokogawa CSU10 Confocal Scanner Unit and an Olympus IX50 microscope with a 1.4NA objective. Images (1280 × 1024) were collected with a Roper Scientific/Photometrics CoolSnap HQ Cooled CCD camera, with a final resolution of 0.11 microns per pixel in the sample plane. A stack of 15 images was collected using a 3 s exposure time and a spacing of 0.5 µm between slices. Stacks were taken every 45 s, with the total period of time for each movie varying depending on the extent of fluorescence photobleaching. Before starting acquisition for each field, the focus was manually adjusted using the GFP fluorescence channel to ensure that cells were centered in the stack.

Time series images were taken for 12 cell lines, each having a different protein labeled with GFP. The proteins (genes) were as follows: cytochrome b-5 reductase (dual) and annexin A5 (anxa5) in cytoplasm, serum deprivation response protein (sdpr) in vesicles and cytoplasm, adipose differentiation-related protein (adip) in vesicles, ADP-ATP translocase 23 (timm23), ATP synthase (atp5a1) and mitochondrial stress-70 protein (hspa9a) in mitochondria, catalase (cat) in mitochondria and vesicles, glucose transporter 1 (glut1) in plasma membrane and t-complex tests expressed 1 (ctt1), alpha-actinin-4 (actn4), caldesmon 1 (cald1) in cytoskeleton.

A major problem with time series images is that photobleaching can occur over time. To minimize the influence of photobleaching, pixel intensities of images at each time point were stretched so that the 95% quantile (and above) was set equal to 256. We chose to stretch the 95% quantile instead of the brightest pixel, because this is less sensitive to noise from artifacts such as fluorescent debris. Figure 3 illustrates the extent of bleaching and correction. Images are preprocessed with background removal and thresholding. For display purposes, only a rectangular region containing a single cell is shown.

2.2 Image preprocessing

Background fluorescence was removed by subtracting the most common pixel value in the image. This was based on the assumption that an image contains more pixels outside the cell than inside it and that background is roughly uniform, both of which hold true for our images. Then a threshold was chosen by an automated method (Ridler and Calvard, 1978), and pixels below the threshold were set to zero. For simplicity, thresholded images were used to calculate all features, although thresholding is not required for calculating texture features. No segmentation was performed because no
We collected 3D time series images, in order to have as much information.

2.4.1 Object tracking features
Calculating features based on object properties, the change is small. The similarity between an object at time

Over time, objects change their positions, shapes and total intensities. These object trajectories.

points, we have the trajectories of objects and we design features based on image to the next. After solving the position of each object in different time

tracking requires several steps. First, we identify all the objects in the image

Similarly, 3D static features were calculated by using the 3D image at the first time point. Thirty-three features that also do not require cell segmentation were chosen from SLF11 (Chen et al., 2004) were used. They include 8 morphological (SLF21.3-SLF1.5

were calculated. 2D Static features were calculated using the center slice at the first time point. Twenty-one of the features from SLF21 (Huang and Murphy, 2004) were used. They include 8 morphological (SLF21.3-SLF1.5 and SLF21.9-SLF21.13) and 13 texture features (SLF21.66-SLF21.78) that are based on the whole image field and do not depend on cell segmentation. Similarly, 3D static features were calculated by using the 3D image at the first time point. Thirty-three features that also do not require cell segmentation were chosen from SLF11 (Chen et al., 2003). They include 5 morphological, 2 edge and 26 texture features.

2.4 2D Temporal feature calculation
In order to set the reference for comparison, both 2D and 3D static features

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2.4.2 Temporal texture features
Temporal texture features were calculated

as described previously (Hu et al., 2006). These were inspired by the Haralick texture features, which capture the intensity correlation of neighboring pixels in space (Haralick, 1979), and designed to capture the value correlation of neighboring pixels in time. We build the temporal gray-level co-occurrence matrix, whose element in row i and column j is the frequency that a pixel with value i changes to value j in the same position at the next time point. Co-occurrence matrices capture information of protein movement, for example, for proteins that show no movement between the two time points, the temporal co-occurrence matrix contains only zeros except on the diagonal.

We calculated 15 statistics described by Haralick (Haralick, 1979) based on the co-occurrence matrix for each pair of images separated by a certain time interval. We used the mean and variance across the time series as the final features of the image. By varying the time interval, we collected 130 features in total: means and variances of the 13 statistics for images 45, 90, 135, 180 and 225 s apart.

2.4.3 Normal flow features
In a series of images taken over time, the intensity of each pixel in each image I (x, y, t) is a function of position x, y and time t. To understand the movement, we need to infer from the observed I (x, y, t), the direction and velocity of each pixel. We use u to represent the vector of movement in the x direction and v to represent the vector of movement in the y direction.

Optical flow is a motion field with vectors (u, v) at each pixel. From the definition, we know (u, v) is pointing to the direction that the pixel moves.
we further assume that the movement is not large. So the right-hand side formula can be approximated with first order:

\[ I(x,y,t+1) = I(x,y,t) + u(x,y) \frac{dt}{dx} + v(x,y) \frac{dt}{dy} \]

Rearranging the equation we have:

\[ u(x,y) \frac{dt}{dx} + v(x,y) \frac{dt}{dy} ] + [ I(x,y,t) - I(x,y,t-1) ] = 0 \]

This is the fundamental equation of motion. \( I_i \) and \( I_j \) are the components of the gradient, which, together with the difference of the intensity \( I_c \) can be directly calculated from the two images. However we are left with one equation with two unknowns (\( u \) and \( v \)). This is the aperture problem: motion along the edge or perpendicular to the gradient can never be recovered. Meanwhile the motion (\( u \), \( v \)) along the gradient is

\[ U_x = \frac{I_x}{\sqrt{I_x^2 + I_y^2}} \]

The flow field containing flows at the gradient direction is called normal flow. Using a, the following 34 features were calculated:

1–13 Haralick texture features of \( U_x \)
14 Mean of \( U_x \)
15 SD of \( U_x \)
16 Mean/SD of \( U_x \)
17–29 Difference of direction from binned direction
30 Mean positive divergence
31 Mean negative divergence
32 Mean positive curl angular velocity
33 Mean negative curl angular velocity

For the texture features of direction, eighteen directions were used. We used 1–8 to represent vectors within the range of 0–44

\[ \sum_{i=1}^{3} \rho X_{i} \frac{\partial X_{i}}{\partial t} + \epsilon \]

where \( \epsilon \) is a constant, \( \rho \) controls how many time points in the past are used in modeling and \( \epsilon \) is estimation error. The \( \rho \) are the parameters we seek to estimate.

We choose to vary \( \rho \) values from 2 to 4, yielding 2, 3 and 4 \( \rho \) values. AR was done on each of the 21 2D static features, resulting in \((2 + 3 + 4)^2 \times 21 = 189\) features.

2.5 3D temporal feature calculation

We expanded the three sets of 2D temporal features to 3D temporal features. Again, only the first 8 time points in each movie were used.

3D temporal texture features were calculated in a similar way as for 2D, except voxels instead of pixels were used when building co-occurrence matrices. A total of 130 features that are based on mean and variance of 13 statistics for five different time intervals, the same as for 2D temporal features, were calculated.

3D normal flow features are defined similarly to 2D. Normal flow on \( x \), \( y \) and \( z \) directions can be calculated with the following equations:

\[ u = \frac{-I_y}{I_x^2 + I_y^2} \]

\[ v = \frac{-I_x}{I_x^2 + I_y^2} \]

\[ w = \frac{-I_z}{I_x^2 + I_y^2} \]

Where \( I_z \) is the voxel intensity difference across time points and \( I_x \), \( I_y \) and \( I_z \) are the gradient projected on the \( x \), \( y \) and \( z \) directions. The speed of a voxel along the gradient is

\[ U_{n} = \frac{I_z}{\sqrt{I_x^2 + I_y^2}} \]

Thirty-three normal flow features were calculated for each pair of 3D images, 16 of which are based on \( I_{n} \), 13 based on Haralick texture features of binned directions and 4 based on divergence and curl (extending the definitions above to three dimensions). A total of 330 features, including mean and variance of features for five different time intervals, were calculated, similarly to the 2D normal flow features.

3D Fourier transform features were calculated similar as in 2D, except that voxels at the same position over time were considered as one time
A well-established SVM package LIBSVM (Chang and Lin, 2001) was used. Therefore, we used stepwise discriminant analysis (SDA) to select the features that have the greatest power to discriminate the classes. While many feature selection methods have been described, SDA has performed well in previous studies of subcellular pattern classification (Huang et al., 2003).

### 2.6 Feature selection

The large numbers of features described above could potentially overwhelm the ability of a classifier to identify meaningful decision boundaries. To further test the power of temporal texture features, the same classification procedure was done without static features. The overall accuracy was 63% with SDA feature selection and 66% without SDA. The confusion matrix without SDA is shown in Table 1. We can see that Cald1 has the lowest classification accuracy (25%).

### 3 RESULTS

By comparing classification accuracy using individual feature sets and combinations of them, we achieved two goals: evaluating the power of each feature set and finding the best way of differentiating our 12 proteins of interest. Three-dimensional time series images are 4D images. Feature sets we have acquired are based on 2D (2D static features of first center slice), 3D across \( z \) (3D static features), 3D across time (2D temporal features of center slices) and 4D images (3D temporal features).

### 3.1 Classification using 2D static features

To set a reference for comparison, classification results of proteins in 3T3 cells were obtained using 21 2D static features. The overall accuracy was 63% with SDA feature selection and 66% without SDA. The confusion matrix without SDA is shown in Table 1. We can see that Cald1 has the lowest classification accuracy (25%).

### 3.2 Classification using 2D temporal feature sets

To evaluate each temporal feature set, we trained classifiers using it with and without the static feature set. When object tracking features were combined with the 2D static features, the average classification accuracies were 61 and 68%, with or without SDA, showing a very small improvement over static pattern classification. From previous work we know when calculating static Haralick texture features, one can change the gray level or resize the image to different resolution and get different classification results (Murphy et al., 2003). Since the same effect would be expected in the temporal domain, we evaluated temporal texture features calculated using different pixel sizes and numbers of gray levels. Classification accuracies are summarized in Table 2. Accuracies fall within the range of 52–71% with SDA feature selection and 66–77% without SDA. The best accuracy of 77% was obtained for 64 gray levels and no downsampling. Apparently, accuracy is higher without SDA, and SVM inherently handled redundancy of features. To further test the power of temporal texture features, the same classification procedure was done without static features. The overall summary of classification accuracy of 21 static combined with 130 temporal texture features, with different gray level and resolution.

<table>
<thead>
<tr>
<th>Resolution (micron/pixel)</th>
<th>Gray level</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>0.11</td>
<td>64.77</td>
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<tr>
<td>1.11</td>
<td>66.67</td>
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</table>

The number before comma is the accuracy in percentage with SDA feature selection, and the number after comma is the accuracy in percentage without SDA.

### Table 1. Confusion matrix for classification using only 2D static features

<table>
<thead>
<tr>
<th>True classes</th>
<th>Prediction by classification</th>
<th>Dia1</th>
<th>Anxa5</th>
<th>Sdpr</th>
<th>Adfp</th>
<th>Timm23</th>
<th>Atp5a1</th>
<th>Hspa9a</th>
<th>Cat</th>
<th>Glu1</th>
<th>Tctex1</th>
<th>Actn4</th>
<th>Cald1</th>
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<tbody>
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<td>Dia1</td>
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<td>45</td>
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<td>5</td>
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<td>5</td>
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<td>Anxa5</td>
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<td>0</td>
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<td>8</td>
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<td>25</td>
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The overall accuracy is 66%.
Table 3. Confusion matrix for the best classification accuracy of 12 protein patterns

<table>
<thead>
<tr>
<th>True classes</th>
<th>Dia1</th>
<th>Anxa5</th>
<th>Sdpr</th>
<th>Adfp</th>
<th>Timm23</th>
<th>Atp5a1</th>
<th>Hepsfu</th>
<th>Cat</th>
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Features used are 2D static, temporal texture, normal flow and Fourier transform features. The overall accuracy is 78%.

classification accuracy was 67% with SDA and 66% without SDA. The results show that temporal texture features themselves capture a significant amount of information.

We next evaluated the normal flow features. Three hundred and forty normal flow features were combined with the 2D static features. Since the normal flow features include texture features calculated on U₀ and the normal flow direction, a similar exploration of number of gray levels and resolution was done. Classification accuracy ranged from 66% to 75% with SDA and 59% to 73% without SDA (data not shown). The best accuracy of 75% was achieved with the original resolution (0.11 μm/pixel) and 64 gray levels, with SDA. When the same classification procedure was done without static features, the overall classification accuracy was 75% with SDA and 74% without SDA. This indicates that these features were more informative than temporal texture features.

When 40 Fourier transform features were combined with the static features, the overall classification accuracy was 69% with SDA and 67% without SDA, a slight increase over using static features alone (data not shown). When using Fourier transform features only, the overall accuracy was 60 and 57% with and without SDA (data not shown).

We next combined 189 autoregression features with the static features. The overall classification accuracy was 59% with SDA and 48% without SDA (data not shown). These are both lower than using static features alone.

From this analysis of each temporal feature set, we found that temporal texture features, normal flow features and Fourier transform features are capable of increasing classification accuracy. We combined these 3 sets of temporal features to search for the best way to differentiate the 12 proteins in 2D time series images. If combined with 2D static features, the overall accuracy is 75% with SDA and 78% without SDA. With temporal features only, very good classification accuracy of 73 and 77% (with and without SDA) can be achieved, proving again the power of the temporal features. The confusion matrix of the best classification is shown in Table 3. Comparing the results with Table 1, the overall accuracy is 12% higher than using just 2D static features. Tctex1 was now classified perfectly. The lowest accuracy of 25% for cald was increased to 56%, and accuracy for Sdpr increased from 56% to 95%. Two other proteins, Anxa5 and Actn4, also have over 20% improvement in accuracy. While the accuracy with SDA selection was lower than without, it is interesting to note that SDA selected 7 static, 15 temporal texture, 13 normal flow and 4 Fourier transform features, from the total of 531 features. This suggests that all of these feature types provide useful information.

3.3 Classification using 3D static features

Static features based on 3D images capture information of images with one more dimension than 2D images. Since we were not able to perform automatic segmentation due to lack of nuclear channel, we selected those features that do not compare to center of cells, thus do not require segmentation. Classification accuracy using 33 field level 3D static features was 74% with SDA, and 71% without SDA. It is 6% higher than using 2D static features and 4% lower than using combined 2D static and temporal features. When 3D static and 2D static features were combined, classification accuracy was 73 and 70%, with and without SDA. As might be expected, the 3D features apparently contain all of the information in the 2D features, and adding redundant 2D static features only made classifier learning more difficult.

3.4 Classification using 3D temporal feature set

Similar to the way we tested 2D temporal features, we combined 3D temporal features with 3D static features to see if the classification accuracy was improved. The result is disappointing. Classification accuracies with or without SDA for 3D temporal features were 74 and 75% for 3D normal flow were 71 and 72% and for 3D Fourier transform were 73 and 71%. Only 3D temporal texture features, combined with 3D static features, performed slightly better than using 3D static features alone. None of the accuracies were higher than the accuracy we achieved combining 2D temporal features with 2D static features. It appears that calculating features over the full 3D images diluted the critical information in the central slices.

4 CONCLUSION AND DISCUSSIONS

Location proteomics as a branch of proteomics study has grown over the last 10 years. To systematically analyze large amounts of data, automatic algorithms have been developed. This article presented
our effort to extend the analysis of static patterns with an additional dimension: the temporal domain. Time series microscopy image of 12 proteins were collected, 5 sets of 2D temporal features and 3 sets of 3D temporal features were implemented and classifications were performed to validate their usefulness. The best 2D temporal feature sets, in the order of their ability to improve classification accuracy, were normal flow, temporal texture and Fourier transform features. Combining 2D static features with 3 sets of 2D temporal feature sets gave the best accuracy of 78%, compared with 66% for static features alone. Accuracy using 3D static and/or temporal features was lower than for 2D features.

If limited acquisition time requires deciding whether to collect 3D static images or 2D time series images, our results suggest that 2D time series images have higher potential of delivering better differentiation. Although not all of the 2D or 3D temporal feature sets improved classification accuracy for our dataset, we still presented them here because they may be useful for future datasets.

While each protein in a proteome is unique, its location patterns might not be. Thus while increasing accuracy of distinguishing the 12 proteins is an indication of feature value, the ability to distinguish them all perfectly is not expected. If proteins interact or co localize with each other, they cannot be differentiated either by static or temporal pattern. In our dataset, alpha-actin-4 (actn4) and caldesmon 1 (cald1) both bind to actin, and over 30% of caldesmon 1 is misclassified as alpha-actin-4. These two proteins are always observed in the same cluster using clustering analysis (data not shown). Similarly, ADP-ATP translocate 23 (timt23) and caspase (cat), which have both been described as mitochondrial proteins, are difficult to distinguish (50% of catalase is misclassified as ADP-ATP translocate 23). The results suggest that there are only 10 distinguishable patterns in our 12 protein set, and this agrees with prior knowledge about these proteins.

For each classification, we compared accuracy with or without SDA feature selection, because SVM is known to be highly robust with large number of correlated features. Our result shows when the number of features is large, 340 normal flow and 189 autoregression, SDA outperforms no feature selection. When the number of features is small, 21 static and 130 temporal textures, SVM does well without SDA feature selection. Many different feature selection algorithms and classifiers could be tried in order to achieve higher classification accuracy, but such an analysis is beyond the scope of this study.

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Since temporal texture features and Fourier transform features can be calculated within 25 and 50 s for each time series, they are readily applicable to many high throughput applications. On the other hand, calculating normal flow features, object tracking features and autoregression features take 8, 22 and 2 min per time series, respectively.

Given the dramatic increase in automated microscopy over the past decade, we anticipate that methods for analyzing temporal changes in protein patterns such as those we have described here will be of significant utility both for basic research in systems biology and for drug screening and development purposes.

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