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Three-Dimensional Spot Detection in Ratiometric Fluorescence Imaging For Measurement of Subcellular Organelles

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Abstract

Lysosomes are subcellular organelles playing a vital role in the endocytosis process of the cell. Lysosomal acidity is an important factor in assuring proper functioning of the enzymes within the organelle, and can be assessed by labeling the lysosomes with pH-sensitive fluorescence probes. To enhance our understanding of the acidification mechanisms, the goal of this work is to develop a method that can accurately detect and characterize the acidity of each lysosome captured in ratiometric fluorescence images. We present an algorithm that utilizes the *h*-dome transformation and reconciles spots detected independently from two wavelength channels. We evaluated our algorithm using simulated images for which the exact locations were known. The *h*-dome algorithm achieved an *f*-score as high as 0.890. We also computed the fluorescence ratios from lysosomes in live HeLa cell images with known lysosomal pHs. Using leave-one-out cross-validation, we demonstrated that the new algorithm was able to achieve much better pH prediction accuracy than the conventional method.

Keywords

Spot detection; *h*-dome transformation; ratiometric imaging; microscopy; lysosomes; pH

1. INTRODUCTION

In endocytosis, waste molecules in the extracellular space are taken up by the cell and degraded by various enzymes in the lysosomes. These enzymes function optimally within a narrow range of acidic pH by which the lysosomes maintain between 4.5 and 5. Therefore, it

is important to understand the mechanisms of lysosomal acidification [1]. One method to quantitatively assess the acidity of lysosomes is to label them with pH-dependent fluorescence probes and image them with a microscope. By dividing the emission intensities obtained from two wavelengths, differences in fluorescence due to factors other than pH can be significantly reduced.

In many studies involving ratiometric imaging, the ratios are derived by applying standard noise reduction techniques followed by intensity thresholding on images from one channel, and the detected pixels are mapped directly to the other channel [2]. Typically, identified areas smaller than a predefined threshold are rejected as noise and the average ratio for each remaining region is used for analysis. Even though a 3-D stack of images is usually acquired, each slice is either processed independently with the possibility of counting a single organelle multiple times or the entire z stack is collapsed into one projection. In addition, a detected region may include more than one object if they are close to each other.

In this paper, we present a new method for 3-D detection of subcellular organelles in ratiometric imaging, taking advantage of having information in three dimensions and from two channels. We have tested the performance of our algorithm on both synthetic images and live HeLa cell images in which the pH of the lysosomes are known.

2. METHODS

2.1 Spot Detection

In the first step of the algorithm, the entire 3-D image stack is transformed based on the h -dome detection method, which has been reported to produce superior performance among a number of spot detection techniques [3]. Initially, particles are enhanced by Laplacian of Gaussian (LoG) filtering ($\sigma_L=5$). An image mask is ρ created by first subtracting the image by a constant h , then performing iterative geodesic dilations until convergence:

$$\rho(I-h)=\lim_{n\rightarrow\infty}\delta_I^{(n)}(I-h). \quad (1)$$

In Eq. 1, I is the LoG-filtered image and δ_I is an elementary dilation operator constrained by I . The domes are extracted simply by subtracting the image mask from the image. Domes with peaks lower than a dynamically-determined threshold are removed, and the intensity-weighted centroids of the remaining domes are reported as the locations of the particles. Our empirical evidence suggests that the value of h chosen does not significantly affect the detection results.

2.2 Determination of Fluorescence Ratios

Traditionally, the lysosomes are detected using one set of images and the same locations are employed on the other set to calculate the fluorescence ratios. To improve accuracy, the algorithm processes the two sets of images independently. For each candidate spot found in one channel, the closest spot detected from the other channel is identified using the quickhull algorithm. If the distance from the closest spot is larger than a certain limit (e.g. T_c

= 4), the point is assumed to be noise and thus rejected. Finally, the emission ratios are calculated using the min-subtracted raw intensity value of the corresponding centroids from the two channels.

3. RESULTS

3.1 Evaluation on Synthetic Images

Synthetic images (Figure 1a) with known ground truth were generated using the BlurLab program. The PSF (Figure 1b) used by the software was derived experimentally from a point-source bead. A minimum distance of one voxel between the spots was imposed as lysosomes cannot overlap with each other. Gaussian noise was added to each dataset to achieve average signal-to-noise ratios (SNR) of 2, 5, and 10, to simulate the levels of noise usually observed in the real images. The fluorescence ratios of the spots between the two channels were fixed to 0.9 or 1.3.

We measured the performance of the algorithm using a commonly applied metric for classification tasks. A spot is considered as a true positive (TP) when its pixel location matches that of an actual particle. The number of false positives (FP) is calculated as the number of predicted spots that are over D_t from the nearest actual spot. The number false negative (FN) is defined as $N_t - TP$, where N_t is the number of actual particles in the data.

The f -score is a measure of the balance between precision ($\frac{TP}{TP+FP}$) and recall ($\frac{TP}{TP+FN}$). As noise level decreases, the f -score increases from 0.655 to 0.890 and 0.553 to 0.862 for the two channels, respectively.

3.2 Evaluation on HeLa Cell Images

HeLa cell images were captured by a wide-field microscope imaging system (Model: Axio Observer.Z1, Carl Zeiss, Germany). For the live cell images, the actual locations of the lysosomes were not known. However, we were able to manipulate the membrane permeability characteristics of the cells so that the lysosomal pH was in equilibrium with the buffered cell culture medium. A range of pHs from 4 to 8 was created. The cells were stained with fluorescein-dextran and Alexa Fluor 555-dextran. Four sets of samples were imaged at each pH level, and each dataset consisted of approximately 10 to 20 slices.

The images were processed using an image processing software called MetaMorph (Molecular Devices, Sunnyvale, CA) and the algorithm proposed here. In MetaMorph, background noise of the images was removed with rank filter subtraction. Using images from one channel, regions with intensities higher than a manually determined threshold were extracted. The average intensity ratio of the pixels within each region was determined by applying the locations detected from one channel to the other channel.

We compared our method to the MetaMorph procedure with leave-one-out cross-validation. In each round, we withheld a different pair of images and generated the pH calibration curve using the others. From the resulting curve, we were able to estimate the pH of the lysosomes

in the hold-out pair. Table 1 shows the average pH prediction error for each pH between the two methods. It also highlights the proportion of fluorescence ratios that cannot be mapped to a pH value as pH ranges only from 0 to 14 and thus defines the boundary of valid fluorescence ratios. Our proposed method is significantly better in both measures, with an average absolute error of 0.370 vs. 0.498.

4. DISCUSSION

While many spot detection methods have been proposed, none seems to tailor to 3-D ratiometric imaging. We propose a new method that employs *h*-dome transformation and maps spots detected independently from the two channels. Comparing to the conventional processing method, the proposed algorithm considers the entire stack of images as a whole, avoids double-counting, and can report the pH of individual lysosomes. We have demonstrated the utility of this algorithm in both synthetic and real cell images for spot detection and pH prediction. Currently, a better spot mapping technique is being developed to more accurately identify corresponding spots from the two channels and to resolve missing detections in either channel.

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Figure 1.
A synthetic image (a) of the lysosomes created using an experimentally-determined PSF shown in (b).

Comparison of pH predicted accuracy on the HeLa cell images using leave-one-out cross validation. Number of spots detected by each method is noted in ().

Table 1

Meth.	Metric	pH 4	pH 5	pH 6	pH 7	pH 8
Old (3241)	Mean Error	0.406	0.349	0.354	0.525	1.383
	% Invalid Pts	89.2%	14.6%	0%	34.6%	46.6%
New (4231)	Mean Error	0.478	0.156	0.237	0.525	0.978
	% Invalid Pts	29.5%	0%	0.19%	29.4%	39.7%