Robust analysis of subcellular, time-lapse microscopy assays

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Introduction

A new generation of microscope and fluorescent probe technologies has enabled highly sensitive quantitative characterization of subcellular objects, such as discrete proteins, organelles, and dendritic spines, in live cell digital microscopy-based functional assays. When approaching the sensitivity limit, the fluorescently labeled subcellular objects often exhibit weak signal that is unstable due to noise and variations. This imposes a critical limitation on the achievable resolution, sensitivity, and characterization accuracy of advanced assay outcomes. Novel image analysis software could enhance signal and normalize noise and variations to overcome this limitation, and extend the achievable sensitivity and accuracy of advanced assay outcomes.

A key first image analysis step associates image points (pixels) to subcellular objects for quantitative characterization of the objects; this step is often referred to as image segmentation or object detection. The conventional image analysis method associates each pixel to either an object or the image background. This binary (black and white) association has inherent error from the digitization effect (a pixel could contain partial object and partial background) that is exacerbated by noise and variations and position shifts over time. For small, subcellular objects the error can become prohibitively large and, therefore, degrade fine measurement and the characterization accuracy and sensitivity of subcellular time-lapse assay outcomes.

We developed a preliminary image analysis method in our microscopy image analysis software, SVCell™, using structure guided processing algorithms[1-7] and confidence mapping. We overcame the inherent error of binary association by making a probabilistic detection of subcellular objects using a confidence score. We performed a feasibility study of this confidence-based method using images from a recycling assay of synaptic vesicles labeled with a fluorescent FM dye. The study results demonstrate that the confidence-based method achieves higher detection and characterization accuracy, and is less susceptible to noise than the conventional method. It is recognized that assay accuracy and sensitivity depend on probe and microscopy technologies. Our results suggest that the quality of image analysis software also plays a critical role in enabling new highly sensitive assays.

Robust object detection method

A digital microscopy image of subcellular objects consists of a set of pixels; each pixel has an intensity value reflecting the content of the image. Due to the digitization effect, the true boundary of an object may not coincide with a pixel boundary and could cover a partial pixel region. The portion of a pixel that is covered could change due to slight position shift in image frame registration.

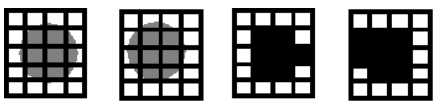

Fig 1. Binary segmentation masks can vary significantly due to a slight shift in image registration

Figure 1(a) and (b) show two identical circles overlaid on a digital pixel grid. The circles are slightly shifted with respect to the grid. Binary segmentation methods would create binary masks in (c) and (d) where the segmentation masks are significantly different even though the difference between the two circles is just a slight shift.

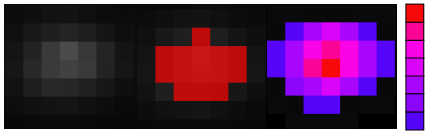

Fig 2. Robust object detection creates a segmentation confidence map rather than a binary mask for each object

Figure 2(a) shows raw grayscale intensity of a subcellular object, in (b) a binary detection mask is overlain onto (a), (c) shows the segmentation confidence for pixels associated with this subcellular object. Segmentation confidence can be derived from spatial information, such as the distance of a pixel to dendritic structure inferred from the pattern of FM dye staining. It can also be derived from each pixel's spatial temporal characterization through the time series.

Once a segmentation mask has been created using the confidence function, it is then used to calculate standard image measurements. For example, median intensity of an object O at time t is usually defined as:

$$m_t = Median\{I(x,y) \mid \forall (x,y) \in O\}$$

where I(x,y) is the intensity value of (x,y). Confidence based median intensity at time t is now defined as:

$$m_{tc} = Median\{C(x,y) * I(x,y) \mid \forall C(x,y) \in O\}$$

where C(x,y) is the refined confidence value of the pixel x,y at time t. Any image measurement can be adjusted to be confidence weighted.

Feasibility study materials and method

Ten images from synaptic vesicle recycling assays using fluorescent FM dye are used for this study. The time-lapse images show the destaining of individual synapses of a small number of rat hippocampal neurons in microisland culture (Figure 3). Each series has 65 images acquired at a rate of 1 Hz. Five baseline image frames were acquired before beginning stimulation. Sixty frames were then acquired during 10 Hz electrical stimulation. The Sullivan lab produced a manual definition of synaptic center and boundaries using SVCell's ROI drawing tool, which was used as a "gold standard truth" for testing.

SVCell results using segmentation confidence masks, here called "confidence" results, were compared with results produced by using binary masks, the "benchmark" results. Binary mask based segmentation was performed using the Image Processing Toolkit (Reindeer Graphics, Inc.) by applying a spatial high pass filter followed by an intensity thresholding.

Feasibility study results

In all tests SVCell confidence-based results were superior and more robust than the benchmark method.

Table 1. The relationship of true and detected objects. The gold standard "truth" is comprised by objects in sets A and C. Confidence or benchmark "detected" objects by either SVCell or the benchmark is comprised by sets A and B. We define the detection rate as D = A / (A+C), and positive predictive value as PPV = A / (A+B).

Detected \ Truth	+	-
+	A	B
-	C	

Detection accuracy study: Detection accuracy is critical to correctly recognize objects in images. The metrics for the detection accuracy study are the detection rate and positive predictive value (PPV). The tests include the comparison of PPV and detection rates for confidence and benchmark methods across the ten test image sets and for four additional sets where zero mean Gaussian noise of standard deviation (sigma) levels 2, 4, 5 and 6 have been added. The estimated average signal level for the ten study images is about 12 grayscale counts, therefore the added noise levels are quite significant. Data here and in subsequent charts show the average metric for individual objects. Error bars show one standard deviation above and below each data point.

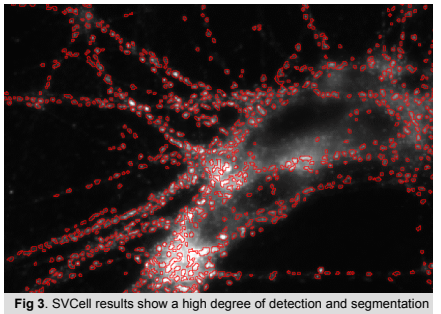

Fig 3. SVCell results show a high degree of detection and segmentation accuracy (high confidence boundary shown)

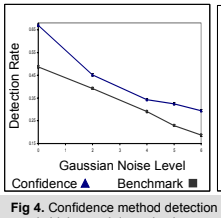

Fig 4. Confidence method detection rate is higher and degrades less with noise (error bars not visible due to large object sample size)

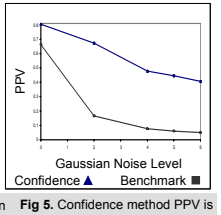

Fig 5. Confidence method PPV is higher and degrades less with noise (error bars not visible due to large object sample size)

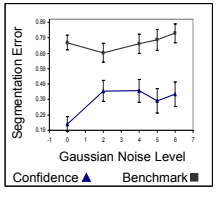

Fig 6. Confidence segmentation error is lower and increases less with noise

Segmentation accuracy study: The metric for the segmentation accuracy study is segmentation error. Segmentation error impacts a system's ability to make accurate measurements and produce high quality assays. For every detected object mask and its corresponding truth mask, the segmentation error is defined as the pixel count of the exclusive OR of the detected and truth masks, divided by the pixel count of the truth mask (limiting the maximum value to 1). The tests include the comparison of the average segmentation error statistics for the SVCell and benchmark results for 5 levels of zero mean Gaussian noise. .

Rate constant (τ) modeling accuracy study: τ is an exponential dissociation model parameter and assay read-out commonly used in synaptic vesicle recycling assays to indicate the rate of neurotransmitter exocytosis from a pre-synaptic terminal loaded with FM dye. Here τ is calculated by fitting single exponentials to the individual synaptic mean intensity data from the time-lapse experiment using nonlinear regression. We expect that improvement in segmentation and measurements would improve not only τ but other model parameters as well.

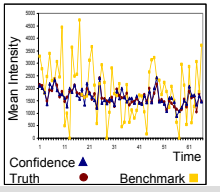

Fig 7. Confidence measurements are closer to the "true" data than the benchmark measurements (single object time plot at noise level 5)

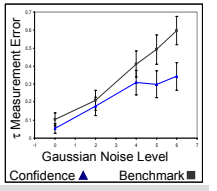

Fig 8. τ measurement error for confidence method is lower and increases more slowly with noise.

Results Summary

The confidence-based method achieves higher synaptic detection rate and positive predictive value that degrades less than the binary mask based benchmark when multiple levels of noise are introduced into the image. The confidence-based method has higher segmentation accuracy and shows less degradation than the conventional approach when exposed to noise. The improved detection and segmentation results yield lower τ measurement error, and the performance degrades less when exposed to noise.

Conclusions

- Robust image analysis methods have been developed for a challenging subcellular time-lapse microscopy assay
- Preliminary results show that robust analysis methods can improve the detection rate and positive predictive value of object detection, the accuracy of object definition, and the accuracy of assay measurements
- We are developing robust kinetic signal and feature measurement algorithms. We expect that these methods will have a direct impact on assay quality and will become a critical enabler for new highly sensitive assays, and will test this hypothesis going forward

Literature cited

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Acknowledgments

This work was supported in part by Grant Number 1 R43 MH075498-01 from the National Institute of Mental Health.