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Evaluation of the effectiveness of Gaussian filtering in distinguishing punctate synaptic signals from background noise during image analysis

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HIGHLIGHTS

- Images of synapses often contain non-specific, spatially broad background noise.
- Background can be defined by Gaussian filtering and subtracted from original image.
- This method was evaluated using hippocampal glutamatergic synapses and simulations.
- This method was efficient in background subtraction and peak detection.
- Some disadvantages were also noted, in comparison to a rolling-ball algorithm.

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ABSTRACT

Background: Images in biomedical imaging research are often affected by non-specific background noise. This poses a serious problem when the noise overlaps with specific signals to be quantified, e.g. for their number and intensity. A simple and effective means of removing background noise is to prepare a filtered image that closely reflects background noise and to subtract it from the original unfiltered image. This approach is in common use, but its effectiveness in identifying and quantifying synaptic puncta has not been characterized in detail.
New analysis: We report on our assessment of the effectiveness of isolating punctate signals from diffusely distributed background noise using one variant of this approach, "Difference of Gaussian(s) (DoG)" which is based on a Gaussian filter.
Results: We evaluated immunocytochemically stained, cultured mouse hippocampal neurons as an example, and provided the rationale for choosing specific parameter values for individual steps in detecting glutamatergic nerve terminals. The intensity and width of the detected puncta were proportional to those obtained by manual fitting of two-dimensional Gaussian functions to the local information in the original image.
Comparison with existing methods: DoG was compared with the rolling-ball method, using biological data and numerical simulations. Both methods removed background noise, but differed slightly with respect to their efficiency in discriminating neighboring peaks, as well as their susceptibility to high-frequency noise and variability in object size.
Conclusions: DoG will be useful in detecting punctate signals, once its characteristics are examined quantitatively by experimenters.

Abbreviations: DIC, differential interference contrast; DoG, difference of Gaussian(s); FWHM, full-width at half maximum; GB, Gaussian-blurring; LED, light-emitting diode; MAP2, microtubule-associated protein 2; PBS, phosphate-buffered saline; ROI, region-of-interest; VGLUT1, vesicular glutamate transporter 1.

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1. Introduction

Sensitive imaging methods for biological and biomedical research are rapidly being developed and refined (Giepmans et al., 2006; Looger and Griesbeck, 2012; Tsien, 2003). Using such methods, the detectable specific signals (positively stained foreground) are often small and well demarcated spatially (i.e., punctate). Nevertheless, the acquired images are often affected by background noise. In neuroscience, for example, fluorescence imaging can identify synapses based on punctate signals obtained by staining for synaptic antigens or functional synaptic markers (Cano et al., 2012; Moulder et al., 2010; Willeumier et al., 2006). Many of these structures are small (e.g. <1 μm) and the diameters of the positive, punctate signals are on the order of <10 pixels when observed by standard light microscopy. In such a scenario, diffuse signals of variable sizes (>10 pixels) and variable intensities are interpreted as background noise of low spatial frequency. The presence of such background noise poses a practical problem, especially when the goal is to distinguish positive signals from noise (segmentation) and to quantify the positive signals that overlap spatially with the background noise, e.g. with respect to number and intensity (Bergsman et al., 2006; Glynn and McAllister, 2006; Harrill et al., 2011; Ippolito and Eroglu, 2010; Schatzle et al., 2012; Stroebel et al., 2010).

Multiple approaches can be used to eliminate background noise. One is to manually assign a single numerical value representing background intensity and subtract it from the original image (Darya et al., 2009; Koh et al., 2002; Schmitz et al., 2011; Zinchuk and Grossenbacher-Zinchuk, 2011). This global-intensity thresholding method (Sahoo et al., 1988) is applicable to cases where the background intensity is uniform across the original image, or across the image after pre-processing. However, this is not often the case; more typically the background noise is variable across an image. Another method for eliminating noises is to divide the image into subregions and to assign and subtract the background intensity value for each subregion. This local-intensity thresholding method (Sahoo et al., 1988) can be performed semi-automatically (Bergsman et al., 2006; He et al., 2003; Lie, 1995). A third way to eliminate noise is to apply a deconvolution method (Agard et al., 1989; Conchello and Lichtman, 2005). This is effective at eliminating out-of-focus noise as well as noise covering an area smaller than the minimal spatial resolution imposed by the imaging method, e.g. the smallest unit that can be resolved optically (point-spread function). If the shape of a positive signal is known, the deconvolution method can be used to retrieve signal information even when multiple signals overlap in clusters (Schmitz et al., 2011). However, the deconvolution method does not effectively remove diffuse background noise. Also its application typically requires detailed knowledge of the imaging method; incomplete knowledge can lead to erroneous data analysis.

In engineering, multiple algorithms are used for removing background noise and detecting signals (Kalaidzidis, 2009). In one simple but effective method, a diffuse background is defined based on smoothing (blurring) of the original image, followed by subtraction of the blurred image from the original. Use of the Gaussian filter for blurring in such applications is the basis of a powerful technique termed "Difference of Gaussian(s)" (DoG) (pp. 291–293 in Russ, 2011). Gaussian functions are typically applied to select objects in a time-dependent series of images, such as those obtained using remote sensing and video surveillance systems (Lee, 2005; Radke et al., 2005; Stauffer and Grimson, 1999, 2000; Wren et al., 1997). Gaussian functions are also applied to select objects in static images (Miettunen and Korhola, 1991; Pal and Pal, 1993). However, the validity of applying Gaussian filtering to biological specimens, and its effects on the intensity, size and detectability of selected objects have not been evaluated systemically and quantitatively. Also it has not been compared with other methods in detail. Therefore the application of this approach is not broadly appreciated among members of the life sciences community. Importantly, there is a paucity of information on how to set the parameters necessary for optimal effectiveness, and how to quantitatively assess a resulting image for accuracy.

Here we report on our characterization of a protocol for DoG and associated image analyses, for removing non-specific background noise. We also provide a quantitative rationale for each step of image analysis. We used immunocytochemically stained, cultured neurons as our samples, and widefield epifluorescence microscopy for imaging. We describe the three fundamental parameters of the image analysis: (1) the radius of the Gaussian-blurring filter (R), (2) the intensity criterion, and (3) the size criterion. We used the ImageJ software, which is freely available in the public domain (http://rsb.info.nih.gov/ij/) and commonly used in the life sciences, and show that the DoG method is simple and semi-automatic, and distinguishes fluorescent puncta from background noise to some extent. We also compare DoG with commonly used methods for removing background (the rolling-ball (Sternberg, 1983) and global-intensity thresholding methods). Given that these methods exhibited different advantages in detecting punctate signals, it will be important for researchers to evaluate and optimize them in the context of the specific characteristics of their imaging applications. Once this is achieved, the methods can be applied to synaptic puncta in any specimen, experiment or signal detection method, provided that the signals of interest are punctate and the background signal is spatially diffuse.

2. Materials and methods

2.1. Ethics statement

Animal care and procedures were approved by the University of Iowa Animal Care and Use Committee (Permit Numbers: 1110226 and 1204080), and performed in accordance with the standards set by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23), revised 1996. Every effort was made to minimize suffering of the animals.

2.2. Culture

Primary hippocampal neurons were cultured from wild-type newborn mouse pups, using a method described in our previous work (Iwabuchi et al., 2013; Kakazu et al., 2012a, 2012b). Briefly, the CA3–CA1 regions of the hippocampus were dissected on postnatal days 0–1, trypsinized and dissociated. The cells were plated on 12-mm coverslips pre-seeded with a rat glial feeder layer (Garcia-Junco-Clemente et al., 2010), in 24-well dishes and at a density of 12,000 cells per well. The feeder layer had been seeded in plating medium of the following composition: MEM (Invitrogen, Carlsbad, CA) plus 5 g/l glucose, 0.2 g/l $NaHCO_3$, 100 mg/l bovine transferrin (EMD Chemicals, Gibbstown, NJ), 2 mM GlutaMAX (Invitrogen), 25 mg/l insulin, and 10% fetal bovine serum (FBS, Invitrogen). Feeder layers were maintained in a 1:1 mixture of plating medium and growth medium. The latter had the following composition: MEM plus 4 μM cytosine β-D-arabinofuranoside, 0.5 mM GlutaMAX, NS21 (Chen et al., 2008) and 5% FBS. The hippocampal neurons were used on day 14 of culture.

The glial cell layers used in these experiments are nearly confluent, with a few patches that are thin and/or the bare glass seems to be directly exposed, as viewed through a light microscope (e.g. lower-right side of magnified DIC image in Fig. 7A). Where the glial layer is present, it can be thick due to the presence of vesicles and cellular processes. This heterogeneity in the glial cell layer can be one contributor to the spatially heterogeneous background noise