Journal of Neuroscience Methods 173 (2008) 83–90

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Automated analysis of secretory vesicle distribution at the ultrastructural level

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ARTICLE INFO

Article history:
Received 17 January 2008
Received in revised form 6 May 2008
Accepted 20 May 2008

Keywords:
Electron microscopy
Large dense core vesicles
Docking
Markov random field
Image processing
Vesicle clustering

ABSTRACT

Neuroendocrine cells like chromaffin cells and PC-12 cells are established models for transport, docking and secretion of secretory vesicles. In micrographs, these vesicles are recognized by their electron dense core. The analysis of secretory vesicle distribution is usually performed manually, which is labour-intensive and subject to human bias and error.

We have developed an algorithm to analyze secretory vesicle distribution and docking in electron micrographs. Our algorithm automatically detects the vesicles and calculates their distance to the plasma membrane on basis of the pixel coordinates, ensuring that all vesicles are counted and the shortest distance is measured. We validated the algorithm on a several preparations of endocrine cells. The algorithm was highly accurate in recognizing secretory vesicles and calculating their distribution including vesicle-docking analysis. Furthermore, the algorithm enabled the extraction of parameters that cannot be measured manually like vesicle clustering.

Taking together, the algorithm facilitates and expands the unbiased and efficient analysis of secretory vesicle distribution and docking.

1. Introduction

One of the key challenges in neuroscience is elucidating the mechanism by which neurotransmitter vesicle secretion is regulated in quantity and in time. Before a neurotransmitter vesicle can be secreted, it has to be transported to the plasma membrane and tightly dock to the location of secretion (reviews by Lin and Scheller, 2000; Sudhof, 2004).

Neuroendocrine cells like chromaffin cells and PC-12 cells are established models for neurotransmitter vesicle transport, docking and secretion (Morgan and Burgoyne, 1997; Neher, 2006). These cells secrete adrenalin and noradrenalin that are both accumulated in large dense core vesicles (LDCVs), which are recognized in electron microscopic pictures by a vesicular membrane containing an electron dense core (Fig. 1) (Plattner et al., 1997; Koval et al., 2001; Unsicker et al., 2005). Using electron microscopy, many studies have been performed to elucidate the molecular mechanism of vesicle transport and docking (Martelli et al., 2000; Ashery et al., 2000; Voets et al., 2001; Sorensen et al., 2003; Kasai et al., 2005; de Wit et al., 2006).

Usually the distribution of LDCVs in micrographs is analyzed manually by measuring the shortest distance of each vesicle to the plasma membrane (Fig. 1B and C). A secretory vesicle is morphologically docked when the vesicular membrane of the vesicle is in direct contact with the plasma membrane. Low magnification micrographs of the entire cell are used to measure cell characteristics such as the plasma membrane length (Fig. 1A). However, these micrographs are usually not of sufficient resolution to discriminate between docked and non-docked vesicles (Fig. 1B and C). Therefore these measurements are performed on multiple partially overlapping micrographs on high magnification. This analysis is a labour-intensive process and subject to human bias and error. For example, vesicles may be counted twice in the overlapping areas and the shortest possible distance has to be determined by the observer.

To solve these problems, we have developed an algorithm to automate the analysis of secretory vesicle distribution and docking in high-magnification electron micrographs. The algorithm generates one stitched master picture of the detail micrographs and automatically detects all secretory vesicles in various endocrine tissue preparations. The distance between the plasma membrane and the vesicle is calculated automatically on the basis of the pixel coordinates, which ensures that the shortest distance is measured. The algorithm is maximally automated to reduce the labour-intensive

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doi:10.1016/j.jneumeth.2008.05.022

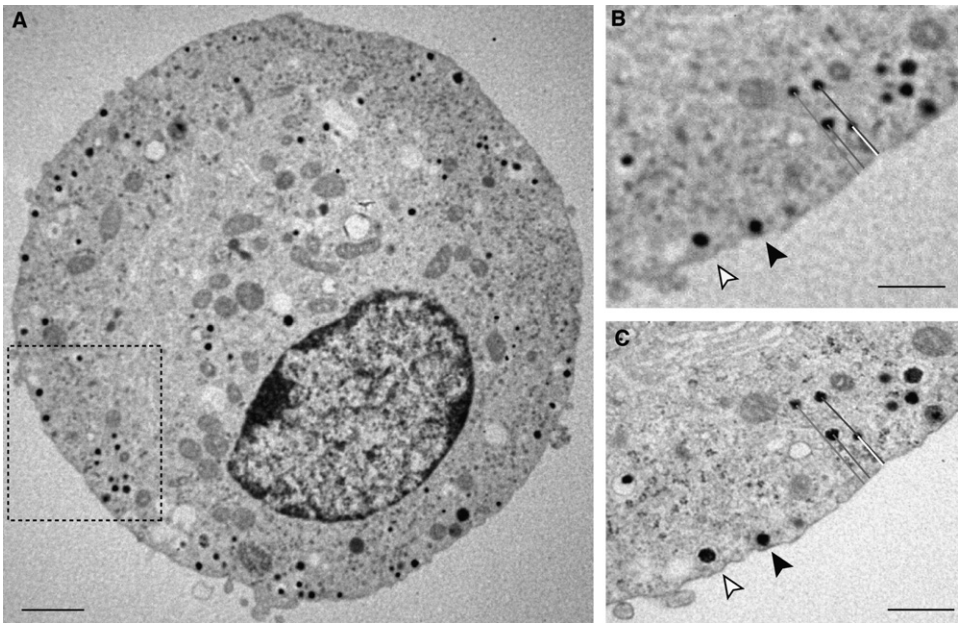

Fig. 1. Low magnification micrographs are not of sufficient resolution to recognize LDCVs and discriminate between docked and non-docked vesicles. (A) A typical example micrograph of an embryonic mouse chromaffin cell in culture at 8000× magnification. Scale bar indicates 1 μm. The dashed rectangle is enlarged in (B). (C) The same region at 20,000× magnification. The high magnification micrograph (C) is, in contrast to the low magnification (B), of sufficient resolution to accurately measure the distance from LDCV to the plasma membrane and discriminate between docked and non-docked LDCVs. Lines show distance from the LDCV centre of gravity to the plasma membrane as measured manually. The black arrowhead indicates a docked LDCV; the white arrowhead indicates a non-docked LDCV. Scale bar indicates 500 nm.

aspect of micrograph analysis and contains a user-interface that makes it available for scientists without programming experience. Furthermore, the algorithm enables the extraction of parameters that cannot be measured manually like vesicle clustering.

2. Materials and methods

2.1. Image acquisition: electron microscopy

Mouse chromaffin cells were obtained at embryonic day 18. Isolated cells were cultured and processed for electron microscopy as previously described (de Wit et al., 2006). Adrenal glands of E18 mice were isolated and fixed in 2.5% glutaraldehyde and 2% formaldehyde in cacodylate buffer. Glands were post-fixed for 2 h with 1% OsO_4/1% $K_4Ru(CN)_6$ in cacodylate buffer and dehydrated through a series of increasing ethanol concentrations. The dehydrated glands were washed in propylene oxide and embedded in Spurr low-viscosity resin. 50 nm sections were stained with 7% uranylacetate and Reynolds lead and imaged by a JEOL 1010 electron microscope at 60 kV. Overview micrographs were obtained at 8000× magnification, detail micrographs at 20,000× magnification. All images were acquired by a Kodak MegaPlus 1.4i camera controlled by analySIS (Soft Imaging Systems/Olympus, Germany).

2.2. Analysis algorithm

The algorithm is designed in the Matlab environment (MathWorks, USA) and is typically provided with one overview micrograph of the entire cell on low magnification and several detail micrographs on high magnification spanning the complete cell section surface, all images in greyscale 8 bit/pixel TIFF format. A general description of the algorithm can be found in the results section, for technical details see supplementary information.

The performance of the algorithm was evaluated in full-automatic and semi-automatic mode. In the semi-automatic mode, the automatically detected LDCVs were scanned by eye by observer 1: obvious false positives were removed and missed LDCVs were added. This corrected set of detected LDCVs was used in the docking evaluation.

Docked LDCVs are defined as those vesicles in direct contact with the plasma membrane. Since the algorithm does not detect the vesicular membrane, it cannot apply this definition. The algorithm assists the observer in docking analysis by marking the group of vesicles in close vicinity of the plasma membrane. This group of automatically assigned LDCVs was checked by observer 1 in semi-automatic docking analysis to judge whether these LDCVs were in direct contact with the plasma membrane.

The described algorithm is available on http://www.synaptologics.nl/databasing.html.

2.3. Manual analysis

The algorithm output was compared to the data of manual analysis performed by three observers using analySIS (Soft Imaging Systems/Olympus, Germany) or ImageJ (NIH, USA). The observers have longstanding experience in manual analysis of secretory vesicles in micrographs and no other instructions were given except the request to count all LDCVs in each cell. The membrane length and cell section surface were measured in low magnification micrographs of the entire cell (8000× magnification) and the number of vesicles were counted in separate detail micrographs (20,000× magnification). Distance to the plasma membrane is measured by the shortest line between the centre of the vesicle and the plasma membrane. Docked LDCVs are in direct contact with the plasma membrane. The manual analysis of total number of LDCVs was performed separately by three expert observers.

Correlations between the datasets were measured by using Pearson product moment correlation coefficient R. Averages were compared by ANOVA and t-test, $p < 0.05$ is considered significantly different. All statistical calculations were performed in Excel (Microsoft Corp., USA).