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Quantitative image analysis tool to study the plasma membrane localization of proteins and cortical actin in neuroendocrine cells

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HIGHLIGHTS

- Automated analysis algorithm for plasma membrane-associated fluorescent signals.
- Applicable to images acquired with confocal and super-resolution microscopy.
- Increased comparability between cells due to automated ROI detection.
- Improved colocalization analysis of membrane associated signals.
- Applicable to live cell imaging data.

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ABSTRACT

Background: Adrenal chromaffin cells are a widely used model system to study regulated exocytosis and other membrane-associated processes. Alterations in the amount and localization of the proteins involved in these processes can be visualized with fluorescent probes that report the effect of different stimuli or genetic modifications. However, the quantitative analysis of such images remains difficult, especially when focused on specific locations, such as the plasma membrane.
New method: We developed an image analysis algorithm, named plasma membrane analysis in chromaffin cells (PlasMACC). PlasMACC enables automatic detection of the plasma membrane region and quantitative analysis of multi-fluorescent signals from spherical cells. PlasMACC runs in the image analysis software ImageJ environment, it is user-friendly and freely available.
Results: PlasMACC delivers detailed information about intensity, thickness and density of fluorescent signals at the plasma membrane of both living and fixed cells. Individual signals can be compared between cells and different signals within one cell can be correlated. PlasMACC can process conventional laser-scanning confocal images as well as data obtained by super-resolution methods such as structured illumination microscopy.
Comparison with existing method(s): By comparing PlasMACC to methods currently used in the field, we show more consistent quantitative data due to the fully automated algorithm. PlasMACC also provides an expanded set of novel analysis parameters.
Conclusion: PlasMACC enables a detailed quantification of fluorescent signals at the plasma membrane of spherical cells in an unbiased and reliable fashion.

Abbreviations: a.u., arbitrary units; BoNT/c, Botulinum toxin C; CC, chromaffin cells; DIV, days *in vitro*; F-actin, actin filaments; LDCV, large dense core vesicle; MCC, mouse chromaffin cell; PBS, phophate buffered saline; PlasMACC, plasma membrane analysis in chromaffin cells; PM, plasma membrane; ROI, region of interest; SIM, structured illumination microscopy; SNARE, soluble N-ethylmaleimide sensitive fusion protein attachment receptor; Syx1A, syntaxin-1A; TIRF, total internal reflection fluorescence.

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1. Introduction

Intercellular communication is highly dependent on fusion of secretory vesicles with the plasma membrane (PM), a biological process referred to as regulated exocytosis. Neuroendocrine cells such as adrenal chromaffin cells (CCs) are an established model system to study regulated secretion of neurotransmitters (Morgan and Burgoyne, 1997). To ensure neurotransmitter exocytosis, the SNARE-complex (Söllner et al., 1993a,b) and a multitude of regulatory proteins (for review see Rizo and Rosenmund, 2008; Mohrmann and Sørensen, 2012; Südhof, 2013) need to be transported to the PM, where they are localized in defined domains. Fig. 1 shows a typical embryonic mouse chromaffin cell (MCC) in primary culture labelled for essential membrane-localized components. Besides regulated exocytosis, a multitude of different essential processes such as endocytosis (Milosevic et al., 2011) occur at the PM. Therefore, biological mechanisms localized at the PM and its adjacent regions need to be studied extensively in order to understand cellular functions.

Our research is focused on the involvement and regulation of the cortical filamentous actin (F-actin) network in neuroendocrine secretion. The quantification of this network in bovine CCs has been subject to a great number of publications, focusing on (de-)polymerization mechanisms (Cheek and Burgoyne, 1986; Vitale et al., 1995; Berberian et al., 2009). Intactness of the cortical network was used as a measure to determine the influence of proteins on regulated exocytosis. Hereby the structural integrity of the actin network was analyzed qualitatively. Previously, we have used a quantitative approach, whereby we manually detected the region of interest (ROI) and used a custom written algorithm to study the intactness of the F-actin network, as well as the absolute number of holes in individual cells (Toonen et al., 2006). To avoid any user-based error due to manual ROI detection, we decided to automate the analysis method. Furthermore, we expanded the set of quantitative parameters to answer additional research questions (*e.g.*, colocalization of proteins and cellular organelles with F-actin and alterations in the filamentous network due to stimulation).

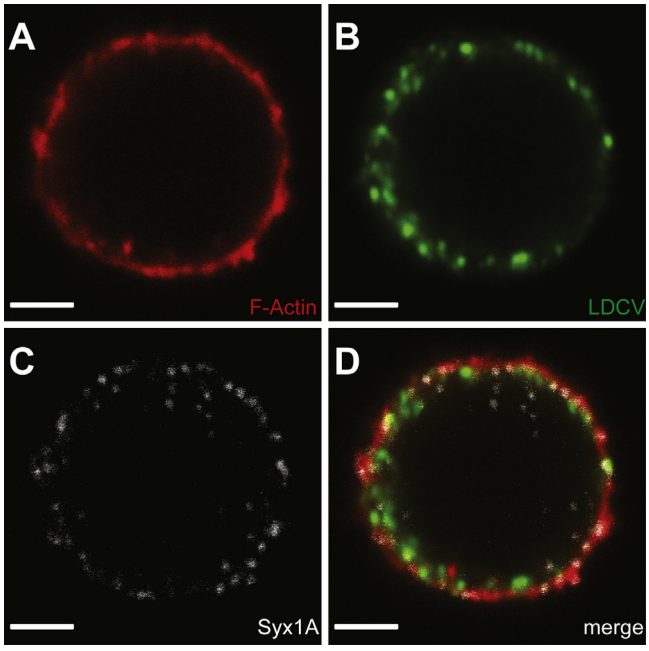

Fig. 1. Equatorial image of a *wild type* embryonal MCC obtained with confocal microscopy and visualizing components of regulated exocytosis: (A) cortical F-actin network stained with rhodamine-phalloidin, (B) LDCVs visualized by a chromogranin A antibody labelling, (C) Syx1A antibody labelling to illustrate the localization of a core player of the exocytosis-machinery, (D) merge of the images; scale bars: 2 μm.

Using immunocytochemical methods or genetic tagging approaches in combination with advanced microscopy techniques, the distribution of proteins, lipids and cellular components can be easily visualized. However, a reliable quantification of the fluorescent signals remains difficult, especially when focused on specific subcellular structures such as the PM. One way to quantify fluorescent signals at the PM is applying linear line scans (De Wit et al., 2006; Rajan et al., 2012; Solis et al., 2012). The measured intensities along the line give an estimation of the ratio of cytosolic and membrane-associated signals. This is a valuable method, but has limitations: since the line intersects the PM at only two points, a large area of membrane-associated signal is not quantified. The placement of a single line can introduce information bias. In earlier publications, we circumvented those limitations by using a circular scan instead of a linear line scan (Toonen et al., 2006). The circular line scan enabled us to quantify fluorescent signals along the entire cell outline. However, with this method we only analyzed the pixels along the manually determined line and still missed valuable information, *e.g.*, the thickness of the signal at the PM or in subplasmalemmal regions.

Plasma sheets can be used as a reduced model system to selectively analyze plasma membrane-localized processes (Avery et al., 2000). The removal of a major part of the cell in these preparations leaves only a part of the PM attached to the coverslip. This PM-fraction, also known as the "footprint" of a cell, can then be analyzed. Those studies delivered valuable information about the composition of the PM and tightly bound components (Lang et al., 2002; Milosevic et al., 2006). Another approach to study membrane-localized mechanisms is total internal reflection fluorescence (TIRF) microscopy (Oheim et al., 1999). An evanescent wave is generated by positioning the excitation laser at a specific incident angle to the coverslip. Thereby only the fluorophores within 200 nm distance from the coverslip are excited and can be observed. One advantage of TIRF microscopy is that the cell is still intact. Another advantage is the increased signal-to-noise ratio of this image acquisition method compared to conventional epi-fluorescence and confocal microscopy. However, TIRF imaging and plasma membrane sheets are limited to the plasma membrane area of the footprint and this area might have a specialized organization. For example, the combination of TIRF microscopy with simultaneous membrane capacitance measurements showed that secretion at the footprint correlates well with the vesicle release of the entire cell, although to a lower extent (10 times lower at the footprint) (Becherer et al., 2007; Hugo et al., 2013). In order to complete our understanding of PM-localized cellular processes, the results obtained by plasma membrane sheet studies and TIRF microscopy should therefore be accompanied by quantitative information of processes on the plasma membrane of the entire cell.

We set out to develop a user-friendly plugin named plasma membrane analysis in chromaffin cells (PlasMACC) that enables a detailed quantification of fluorescent signals at the plasma membrane in an unbiased and reliable fashion and runs in the image analysis software ImageJ environment (http://rsb.info.nih.gov/ij/). We have briefly described an earlier version of this method (Kurps and De Wit, 2012). Now, after testing and improving our algorithm thoroughly and extending its applications, we make it available for a wider community. PlasMACC is compatible with data acquired by a wide variety of fluorescence microscopy techniques and permits an objective, highly reproducible and quantitative study of fluorescent signals at the PM of neuroendocrine cells. Furthermore, PlasMACC enables the user to address more complex research questions regarding colocalization of cellular components and stimulation dependent changes.