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Colocalization of synapse marker proteins evaluated by STED-microscopy reveals patterns of neuronal synapse distribution *in vitro*

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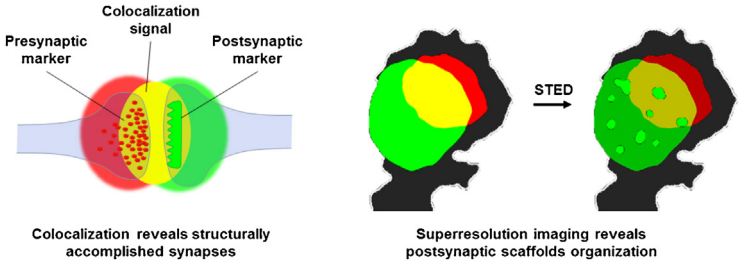
HIGHLIGHTS

- A reliable and easy-to-use workflow for synapse density quantification is presented.
- The differential distribution of GABAergic and glutamatergic synapses is assessed.
- Super-resolution STED-imaging of the postsynapse reveals scaffolding proteins clusters.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Quantification of synapses and their morphological analysis are extensively used in network development and connectivity studies, drug screening and other areas of neuroscience. Thus, a number of quantitative approaches were introduced so far. However, most of the available methods are highly tailored to specific applications and have limitations for widespread use.

New method: We present a new plugin for the open-source software ImageJ to provide a modifiable, high-throughput and easy to use method for synaptic puncta analysis. Our approach is based on colocalization of pre- and postsynaptic protein markers. Structurally completed glutamatergic and GABAergic synapses were identified by VGLUT1-PSD95 and VGAT-gephyrin colocalization, respectively.

By combining conventional confocal microscopy with stimulated emission depletion (STED) imaging, we propose a method to quantify the number of scaffolding protein clusters, recruited to a single postsynaptic density.

Results: In a proof-of-concept study, we reveal the differential distribution of glutamatergic and GABAergic synapse density with reference to perineuronal net (PNN) expression.

Using super-resolution STED imaging, we demonstrate that postsynaptic puncta of completed synapses are composed of significantly more protein clusters, compared to uncompleted synapses.

Comparison with existing methods: Our Synapse Counter plugin for ImageJ offers a rapid and unbiased research tool for a broad spectrum of neuroscientists. The proposed method of synaptic protein clusters

Abbreviations: GABA, γ-aminobutyric acid; Geph, gephyrin; PNNs, perineuronal nets; PSD95, postsynaptic density protein 95 kDa; STED, stimulated emission depletion; VGAT, vesicular γ-aminobutyric acid transporter; VGLUT1, vesicular glutamate transporter.

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quantification exploits super-resolution imaging to provide a comprehensive approach to the analysis of postsynaptic density composition.
Conclusions: Our results strongly substantiate the benefits of colocalization-based synapse detection.

1. Introduction

Synapse quantification approaches aim to improve our understanding of how neuronal networks are established and modified. As main means of signal transduction, synapses provide an adjustable machinery to connect neurons within the brain (Lee et al., 2016; Sigal et al., 2015; Womelsdorf et al., 2014). The distribution of synapses and their morphology undergoes substantial changes during neuronal maturation and in neuronal plasticity in response to pathophysiological stimuli (Faissner et al., 2010; Frischknecht et al., 2009; Geissler et al., 2013). For this reason, a method for efficient and reliable synapse quantitation is of clear interest for both fundamental and applied neurobiological studies.

Synaptic puncta are clusters of presynaptic and postsynaptic marker proteins that are formed during synapse formation and maturation. Here we propose a new easy, fully automated, user-independent ImageJ plugin for rapid quantification and size estimation of synaptic puncta called "Synapse Counter". We further combine the same algorithm for processing confocal images with STED microscopy to resolve the ultrastructure of postsynaptic puncta. Thus, we quantify the number of clusters of postsynaptic proteins, as resolved by STED microscopy, within the fluorescent postsynaptic puncta spots, detected by confocal microscopy. Importantly, within this approach, both GABAergic and glutamatergic synapses are defined in a similar way, which allows the comparison between excitatory and inhibitory inputs within a network. This aspect is especially relevant for excitation-inhibition balance studies.

Super-resolution microscopy techniques can be efficiently used to visualize the ultrastructural organization of synapses (Kittel et al., 2006; Maglione and Sigrist, 2013; Owald and Sigrist, 2009). In this work, we used STED-microscopy with time-gated detection (Vicidomini et al., 2013) to reveal the subtle organization of postsynaptic scaffolds. Furthermore, we propose a straightforward method to quantify the complexity of these assemblies.

As a proof of concept study, we examined the distribution patterns of glutamate- and GABAergic synapses with reference to perineuronal net (PNN) expression. PNNs are complex macromolecular assemblies that are enriched around the somata and proximal dendrites of several types of neurons. These structures are predominantly expressed by parvalbumin containing interneurons and represent a specialized condensed form of the extracellular matrix (for review see (Deepa et al., 2006; Kwok et al., 2011)). Interestingly, these assemblies have a peculiar role in synaptogenesis and neural plasticity regulation (for review see (Dzyubenko et al., 2016; Faissner et al., 2010; Frischknecht and Gundelfinger, 2012)). In this work, we asked whether the presence of PNNs around the somata of hippocampus neurons associates with distinguishable synapse formation patterns in PNN-positive as compared to PNN-negative neurons.

2. Materials and methods

2.1. Ethical standards

The present study was carried out in accordance with the European Communities Council Directive of September 22, 2010 (2010/63/EU) for care of laboratory animals. All efforts were made to reduce the number of animals used.

2.2. Animal housing

Male and female NMRI mice were used in all conducted experiments. Mice were housed individually with a constant 12-h light-dark cycle and access to food and water *ad libitum*.

2.3. Cell cultures

Indirect cocultures of neurons and astrocytes were obtained and maintained as described previously (Geissler et al., 2013; Pyka et al., 2011a, 2011b). Briefly, hippocampal neurons were derived from the embryos of NMRI mice at 15.5 days post conception, and cortical astrocytes were obtained from newborn NMRI mice (postnatal day 1–3). Primary neurons and astrocytes were cultivated separately, but sharing the same medium. The neurons were cultivated on glass coverslips on the bottom of 24-well companion culture dishes (BD Biosciences) at the density of 20,000 cells/cm^2. Astrocyte monolayers were provided to neurons using cell culture inserts (pore size 0.4 μm; BD Biosciences), thus creating indirect astrocyte and neuron cocultures, which allow for long-term experiments. After reaching the mature state at 21 days in vitro, the cultures were processed for immunocytochemistry.

2.4. Immunocytochemistry

For immunofluorescence labeling of synaptic markers, neuronal markers and perineuronal nets, the cultures were fixed with 4% w/v paraformaldehyde (Sigma-Aldrich) for 10 min at room temperature. To prevent the unspecific binding of antibodies, the samples were incubated for one hour at room temperature with 30% v/v normal goat serum (Jackson Immuno) in Antibody Dilution Solution (AbDil: 150 mM NaCl, 50 mM tris-base, 1% w/v BSA, 100 mM L-lysine, 0.05% v/v Triton-X100, pH 7,4). After washing twice with PBS, primary antibodies were applied in AbDil for 14 h at 4 °C. For presynaptic puncta detection, the following antibodies against vesicular transporters of correspondent neurotransmitters were used: anti-VGLUT1 (1:500, polyclonal from guinea pig, Synaptic Systems) for glutamatergic axonal terminals, and anti-VGAT (1:500, polyclonal from guinea pig, Synaptic Systems) for GABAergic axonal terminals. To identify postsynaptic puncta, the antibodies against postsynaptic scaffolding proteins were applied: anti-PSD95 (1:500, mouse clone 7E3-1B8, Millipore) for excitatory synapses, and anti-gephyrin (1:500, mouse clone mAb7, Synaptic Systems) for inhibitory synapses. Perineuronal nets were identified on the base of the intense aggrecan staining (anti-aggrecan, polyclonal from rabbit, Millipore). Parvalbumin-containing interneurons were labeled with anti-parvalbumin antibodies (1:500, polyclonal from chicken, Synaptic Systems). Secondary antibodies anti-mouse IgG Alexa 488 (Jackson Immuno) were used to detect PSD95- and gephyrin-, anti-rabbit IgG Alexa 546 (Jackson Immuno) for aggrecan, anti-chicken IgY Alexa 488 (Jackson Immuno) for parvalbumin, and anti-guinea pig IgG Alexa 633 (Jackson Immuno) for VGLUT1- and VGAT-antibodies. Secondary antibodies were applied in Abdil with 5% v/v normal goat