Advanced STORM super-resolution techniques for astrocyte-specific molecular imaging

Summary of the PhD Thesis

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Budapest
2026

1. Introduction

The paradigm of the "tripartite synapse" has elevated astrocytes from passive supportive elements to active modulators of neurotransmission and brain homeostasis. As a result, astrocytes have emerged as key therapeutic targets for neurodegenerative and neuroinflammatory disorders. However, elucidating the precise distribution of astrocytic proteins lags significantly behind neurons, presenting a major barrier to understanding molecular mechanisms within the central nervous system.

A prominent target that underscores the need for such molecular mapping is monoacylglycerol lipase (MAGL). Operating at the crossroads of retrograde endocannabinoid and inflammatory signaling, MAGL serves as the primary clearing mechanism for the endocannabinoid 2-arachidonoyl-glycerol (2-AG), while simultaneously providing the precursor pool for downstream pro-inflammatory prostaglandin synthesis. Notably, cell type-specific knockout studies have revealed that this coupling of lipid mediators is driven predominantly by astrocytic, rather than neuronal, MAGL. Importantly, targeting astrocytic MAGL provides robust neuroprotection in models of brain injury and

neurodegeneration without triggering the adverse behavioral side effects or tolerance typical of global cannabinoid therapies.

Spatially distinct subcellular pools of the enzyme are hypothesized to independently regulate these diverse pathways. However, mapping MAGL within cortical and hippocampal astrocytes has been severely hindered by technical limitations. Because astrocytic MAGL possesses a remarkably low copy number, electron microscopy is heavily constrained by low structural accessibility and suboptimal labeling efficiency for such scarce targets. On the other hand, conventional light microscopy lacks the spatial resolution required to isolate these faint signals from dense surrounding structures.

This latter challenge is fundamentally rooted in the highly complex morphology of astrocytes. Their perisynaptic processes possess dimensions well below the 250 nm diffraction limit of conventional fluorescence microscopy. To overcome this barrier, Stochastic Optical Reconstruction Microscopy (STORM) has emerged as a powerful single-molecule localization technique. By utilizing the stochastic photoswitching of fluorophores, STORM bypasses the diffraction limit to achieve nanoscale resolution down to tens of nanometers, making it uniquely suited to resolve the fine

astrocytic nanoarchitecture. Furthermore, its single-molecule sensitivity allows for the detection and quantification of low-copy, scarce targets within native tissue structure.

2. Objectives

Elucidating the precise subcellular distribution of MAGL is critical to unlocking its therapeutic potential in neuroinflammatory disorders. Because dense, overlapping signals in brain tissue make it challenging to separate astrocytic and presynaptic enzyme pools, this study introduces a nanoscale imaging framework designed to map individual astrocytes.

To achieve this, our project was divided into three distinct aims:

2.1 **Astrocytic labeling strategy (PALE):** To develop and implement a sparse, membrane-targeted expression strategy in individual astrocytes via postnatal electroporation, a method not yet established for hippocampal astrocytes. This approach provides a clear fluorescent scaffold to resolve the finest astrocytic arbors using STORM-compatible dyes.

2.2 **Dual-color STORM imaging controls:** To implement rigorous control measurements verifying that channel crosstalk and chromatic aberration do not interfere with target localization, ensuring faithful spatial discrimination

between astrocytic filaments and adjacent synapses.

2.3 **Nanoscale mapping of astrocytic MAGL:** To determine whether MAGL is preferentially enriched near hippocampal CA1 synapses or follows a random distribution, utilizing advanced tessellation approaches and molecular distance calculations.

3. Methods

3.1 Animals used in this study. Mice were kept under approved, specific-pathogen-free laboratory conditions, and all efforts were made to minimize pain and to reduce the number of animals used. To study the nanoscale distribution of MAGL and to verify the specificity of the MAGL antibody, we used male *Mgll*$^{+/+}$ and *Mgll*$^{-/-}$ littermates that were kept on C57BL/6N background. To develop the methodology for the PALE approach and astrocyte-specific STORM imaging in the CA1 subregion of the hippocampus, we used male and female CD-1 mice.

3.2 Postnatal Astrocyte Labeling by Electroporation (PALE). Newborn and postnatal day 1 (P0-P1) pups were sedated by hypothermia, and plasmid DNA solution was injected into one of their lateral ventricles. Electroporation was performed with tweezer electrodes using an SP-3c electroporator. We used the membrane-targeting constructs pAAV-GFAP104-ChR2-mCherry and Lck-GFP.

3.3 Preparation of hippocampal sections. Two approaches were used in this study. In the first, mice were perfused transcardially with 4% paraformaldehyde (PFA), brains were

removed, and hippocampal coronal sections were cut on a Leica VT-1200S vibratome at thicknesses of 20 μm for STORM imaging and 50 μm for confocal microscopy. In the second approach, acute hippocampal slices were prepared in the same manner as the traditional approach in *ex vivo* electrophysiological experiments. Briefly, mice were deeply anesthetized by isoflurane, the brains were quickly removed from the skull, and transferred to ice-cold sucrose containing artificial cerebrospinal fluid. The 300-μm-thick coronal hippocampal acute slices were cut using a vibratome and were placed into 4% PFA for overnight immersion fixation. Slices containing a suitable number of astrocytes (as determined via an upright fluorescence microscope) were re-embedded in 2% agarose and cut on a vibratome to 20-μm-thick sections.

3.4 Immunolabeling. Immunolabeling of hippocampal sections was carried out in a free-floating manner in tissue culture plates. Sections were blocked and permeabilized with 5% normal donkey serum (NDS) and 0.3% Triton in Tris-buffered saline (TBS) for 45 min, then incubated overnight at 4 °C in primary antibody solution diluted in TBS. Incubation in the respective secondary antibody solution was carried out for 4 hours. For

super-resolution imaging, the sections were postfixed with 4% PFA and mounted onto coverslips.

3.5 STORM image acquisition. Dried sections mounted on coverslips were covered just prior to imaging in STORM imaging buffer containing thiol-based switching agent and oxygen-scavenging system. We used a Nikon N-STORM system that was equipped both with a C2 confocal scan head and an Andor iXon Ultra 897 EMCCD camera and was operated by the NIS Elements AR 4.3 software and N-STORM 3.4 module.

3.6 STORM image analysis. Peak detection of individual localization events was performed by the N-STORM module. Custom written Python codes were used in conjunction with various software to analyze the STORM data. To assess the level of colocalization of dual-color STORM images, we retrieved the Manders' coefficient values from Voronoi tessellations using Coloc-Tesseler. To filter MAGL localization points belonging to astrocytes, we created 2D Voronoi cells from the astrocytic localization points in SR-Tesseler. To calculate nearest neighbor distances, we used the SciPy module in Python. The DBSCAN algorithm was used to identify localization point clusters.

3.7 Correlated confocal and STORM imaging. A confocal z-stack was acquired from the very same region of interest that was subsequently imaged by the STORM microscope. Confocal images were deconvolved with Huygens software (SVI) using the CMLE algorithm. The two different imaging modalities were aligned in the VividSTORM software. The confocal images were thresholded, and the binary image was converted into a point-coordinate list to enable distance calculations in the STORM image's coordinate system.

4. Results

4.1 Visualizing the intricate architecture of hippocampal astrocytes at the nanoscale level.

By modifying the PALE strategy, we could selectively and sparsely label hippocampal astrocytes. All labeled cells showed the characteristic spongiform morphology of astrocytes, and immunolabeling experiments verified that all cells expressed the astrocytic marker glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS) but not the neuronal marker NeuN or microglial marker Iba1. This approach appeared to be highly reproducible, with 74% of electroporations yielding more than 10 cells in a 20 μm thick section. As opposed to GFAP, the electroporated membrane-targeting fluorescent proteins visualized the full extent of astrocyte morphology.

Immunolabeling of fluorescent proteins with antibodies carrying STORM-compatible dyes enabled us to reconstruct the astrocytic arbor at the nanoscale level. We observed loop-like structures that appeared to be non-continuous only when imaging with STORM, but not confocal, modality. The spongiform astrocytic domains showed narrower and wider sections alternating on the very same segments. Intensity

measurements along some narrower sections showed that their widths as quantified by the full width at half maximal values are well below the diffraction limit, and hence confocal microscopy falsely enlarges these structures.

4.2 Dual-color STORM imaging of hippocampal astrocytes in brain tissue.

First, we evaluated the degree of channel crosstalk on our microscope setup. We labeled astrocytes either with Alexa Fluor 647 (AF647) or CF568 fluorophores, and imaged them either with their specific, or non-specific laser line (647 nm or 561 nm, and vice versa). The 561 nm laser line also excited AF647 fluorophores and resulted in a dim blinking that could be eliminated by applying more stringent peak identification settings. The 647 nm laser line did not excite CF568 fluorophores – hence we choose to label our target of interest with AF647 fluorophores, which gave superior blinking characteristics compared to CF568.

Next, we determined the level of chromatic aberration by labeling the very same astrocytes with two different secondary antibodies, carrying either CF568 or AF647 fluorophores. To quantify the degree of overlap we used Coloc-Tesseler that relies

on the Voronoi tessellation method to compute the Manders' coefficient value directly from the molecular coordinates and their local densities. Individual dual-labeled astrocytes showed a very high degree of overlap and shifting one STORM channel relative to the other started to deteriorate the Manders' values.

Then, we visualized two targets at the nanoscale level alongside with the astrocytic arbor: glutamine synthetase (GS), a cytosolic protein specific to astrocytes; and Bassoon, a presynaptic active zone scaffold protein located in neuronal axon terminals. Dual-color STORM images revealed that GS localization points completely overlapped with the PALE localization points, while Bassoon clusters were often adjacent to perisynaptic astrocytic processes but still well separated without any apparent co-localization. Quantifying the Manders' coefficients and nearest neighbor distances (NND) of the target proteins to the astrocytic localization points verified our visual observations. Notably, image rotations were used as controls, further corroborating our results that the observed data are not coincidental.

4.3 Examining the nanoscale distribution of MAGL in astrocytes.

we carried out PALE on MAGL wild type (WT) and MAGL knockout (KO) pups, and performed dual-color STORM imaging of MAGL and astrocytes labeled by PALE. By tessellating the PALE STORM signal and by setting a density threshold we created objects with contours delineating the astrocytic arbor. These contours then served us as region of interests (ROIs) to classify MAGL localizations as either astrocytic or non-astrocytic (possibly synaptic) ones. Interestingly, we found that the densities of both non-astrocytic and astrocytic MAGL localization points were much higher in WT compared to KO sections. Notably, astrocytic localization points in a given neuropil area accounted only for 14% of all localizations.

Next, we compared the Ripley's *H* function of MAGL STORM images to that of Bassoon to show that MAGL in astrocytes is likely present in a scattered, non-clustered pattern, and only the multiple on-off switching cycles of STORM imaging creates the clustered MAGL localization point patterns. Comparing the spatial characteristics of these astrocytic and non-astrocytic point patterns did not yield any statistical difference.

Lastly, we explored if astrocytic MAGL is located preferentially near synapses. To this end, we measured the nearest neighbor

distances of astrocytic MAGL to active zones marked by Bassoon (using correlated confocal and STORM microscopy) and compared their distribution to randomized datasets. As opposed to the highly abundant non-astrocytic MAGL localization points in the vicinity of Bassoon, astrocytic localization points did not exhibit any spatial bias toward synapses.

5. Conclusions

In this doctoral thesis, I presented a nanoscale imaging framework to visualize functional proteins within individual astrocytic processes. The utility and precision of this method were demonstrated by mapping the distribution of the endocannabinoid-degrading enzyme, monoacylglycerol lipase (MAGL), specifically within astrocytes.

The core conclusions of this work are summarized as follows:

- **Targeted astrocytic labeling:** Postnatal electroporation provides a robust method to sparsely label hippocampal astrocytes. This approach enables complete, individual morphological reconstructions using STORM super-resolution microscopy.

- **Dual-color STORM imaging of astrocytes:** High-quality dual-color STORM imaging requires strict calibration to control for chromatic aberration and channel crosstalk. Combining nearest neighbor distance and co-localization metrics with image rotation control measurements provides a powerful, verified toolset to

retrieve cell-specific molecular positions within the tripartite synapse.

- **Synapse-independent spatial distribution of astrocytic MAGL:** Voronoi tessellation-based object creation successfully discriminated between astrocytic and non-astrocytic pools of MAGL. Correlated confocal and STORM imaging revealed that astrocytic MAGL lacks spatial preference toward synaptic release sites, suggesting a functional role independent of direct synaptic endocannabinoid regulation.

Taken together, these results underscore the necessity of high-resolution, single-molecule methods to map clinically relevant astrocytic pathways at the nanoscale.

6. Bibliography of the candidate's publications

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