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4 Probing nano-organization of astroglia with multi-color super-
5 resolution microscopy
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ABSTRACT

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Astroglia are essential for brain development, homeostasis and metabolic support. They also contribute actively to the formation and regulation of synaptic circuits, by successfully handling, integrating and propagating physiological signals of neural networks. The latter occurs mainly by engaging a versatile mechanism of internal Ca^{2+} fluctuations and regenerative waves prompting targeted release of signaling molecules into the extracellular space. Astroglia also show substantial structural plasticity associated with age- and use-dependent changes in neural circuitry. However, the underlying cellular mechanisms are poorly understood, mainly because of the extraordinary complex morphology of astroglial compartments on the nanoscopic scale. This complexity largely prevents direct experimental access to astroglial processes, most of which are beyond the diffraction limit of optical microscopy. Here we employed super-resolution microscopy (direct stochastic optical reconstruction microscopy; dSTORM), to visualize astroglial organization on the nanoscale, in culture and in thin brain slices, as an initial step to understand the structural basis of astrocytic nano-physiology. We were able to follow nanoscopic morphology of GFAP-enriched astrocytes, which adapt a flattened shape in culture and a sponge-like structure *in situ*, with GFAP fibers of varied diameters. We also visualized nanoscopic astrocytic processes using the ubiquitous cytosolic astrocyte marker proteins S100 β and glutamine synthetase. Finally, we overexpressed and imaged membrane-targeted pHluorin and lymphocyte-specific protein tyrosine kinase-green fluorescent protein (Lck-GFP), to better understand the molecular cascades underlying some common astroglia-targeted fluorescence imaging techniques. The results provide novel, albeit initial, insights into the cellular organization of astroglia on the nanoscale, paving the way for function-specific studies.

SIGNIFICANCE STATEMENT

Astroglia are a critical contributor to the development and function of brain circuits. However, the physiological basis of diverse astroglial activities remains largely an enigma, mainly because of the poor experimental access to the nanoscopic astroglial compartments filling the tissue volume. Super-resolution microscopy could thus provide an important tool to understand molecular organization of astrocytes on a small scale. Here we outline results of our initial quests to follow nano-organization of astroglial processes using super-resolution imaging of astroglia associated proteins GFAP, S100 β and glutamine synthetase. These probes provide initial insights into the nano-organization of structurally intact astroglial on the scale from nanometers to microns, which has hitherto been unattainable.

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INTRODUCTION

Astrocytes have emerged as an active neural circuit participant contributing to the information processing in the brain. They are equipped with numerous plasma membrane receptors, transporters and ion channels to enable receipt and transduction of diverse physiological inputs from the brain networks, in health and disease (reviewed in (Agulhon et al. 2008; Bennett et al. 2012; Dityatev and Rusakov 2011; Halassa and Haydon 2010; Haydon 2001; Matyash and Kettenmann 2010; Porter and McCarthy 1997; Seifert et al. 2010; Verkhratsky et al. 2012; Volterra and Meldolesi 2005)). Electrically non-excitable astroglia appear capable of integrating and communicating such physiological signals through the versatile machinery of intracellular calcium sparks, elevations and regenerative waves that exhibit wide-ranging spatiotemporal modalities across different cellular compartments (recently reviewed in (Araque et al. 2014; Bazargani and Attwell 2016; Khakh and Sofroniew 2015; Rusakov 2015; Volterra et al. 2014a; Zorec et al. 2012)). These Ca^{2+} fluctuations are translated by astrocytes into the release of signaling molecules, or gliotransmitters, - such as D-serine, glutamate, adenosine/ATP, α -TNC - triggering distinct functional responses in neurons (see references above).

This functional diversity coupled with the nearly universal Ca^{2+} dependence suggests that astroglia could operate specialized functional cellular compartments, on the microscopic or nanoscopic scale (Rusakov et al. 2014). In this context, much attention has been paid to ultrathin astrocyte processes that surround excitatory synapses (perisynaptic astrocytic processes, PAPs) and thus seem to provide a local cellular substrate for intimate astroglia-synapse signal exchange - most notably through glutamate transport and potassium buffering (Bergles et al. 1999; Danbolt 2001; Heller and Rusakov 2015; Rusakov et al. 2014) but also through the release of gliotransmitters. PAPs are present throughout the brain providing variable astroglial coverage of synapses depending on the region, the synaptic identity, but also on local circuit activity (Bernardinelli et al. 2014b; Haber et al. 2006; Heller and Rusakov 2015; Hirrlinger et al. 2004; Perez-Alvarez et al. 2014; Theodosis et al. 2008).

The cellular basis of versatile astroglial activity, and whether it involves specialized cellular compartments such as PAPs, remains poorly understood (recently reviewed by in (Bernardinelli et al. 2014a; Heller and Rusakov 2015)). This is mainly because direct experimental probing of the nanoscopic astrocyte structures has not been technically plausible: the sponge-like astrocyte processes fill the tissue volume featuring details that are beyond the diffraction limit of light microscopy (200-300 nm, including fluorescence confocal and two-photon excitation varieties). Historically, electron microscopy (EM) has been the only tool capable of successfully resolving astroglial

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3 structure on the nanoscale, including advanced 3D reconstruction tools to deal with
4 PAPs (Bernardinelli et al. 2014c; Lushnikova et al. 2009; Medvedev et al. 2014;
5 Medvedev et al. 2010; Patrushev et al. 2013; Popov et al. 2004; Witcher et al. 2007;
6 Witcher et al. 2010). However, EM has important limitations. Firstly, the extensive
7 human and instrumental resources involved make it unfeasible to deal with more than a
8 very few microscopic cell fragments in an individual 3D EM study. Correspondingly,
9 obtaining a contiguous macroscopic pattern for the molecule of interest (receptor,
10 channel, transporter, etc.) at nanoscopic resolution using EM will be technically
11 challenging if at all feasible. In other words, EM has a limited ability to provide
12 nanoscopic information in the context of the macroscopic organization of the cell and
13 the surrounding tissue. Recent advances in super-resolution microscopy appear to
14 overcome some of these deficiencies providing resolution of up to 10-70 nm while
15 imaging structurally intact cells (Bates et al. 2007; Betzig et al. 2006; Huang et al. 2008;
16 Klar et al. 2000). Applications of stimulated-emission depletion (STED), photo-activated
17 localization microscopy (PALM) or stochastic optical reconstruction microscopy
18 (STORM) are beginning to reveal some important aspects of astroglial nano-
19 organization (Heller and Rusakov 2015; Panatier et al. 2014; Rossi et al. 2012; Smith
20 and Verkman 2015; Volterra et al. 2014b).

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22 Here, we adapt a direct STORM (dSTORM) technique, which uses stochastic excitation
23 of sparsely distributed fluorophores (Endesfelder and Heilemann 2015; van de Linde et
24 al. 2011), in an attempt to understand some basic aspects of the structural and
25 molecular organization of astroglia on the nanoscale. We chose to image glial fibrillary
26 acidic protein (GFAP), a common astroglia marker (Oberheim et al. 2012), a key
27 astroglial metabolite glutamine synthetase (Derouiche and Frotscher 1991; Volterra et
28 al. 2014b) and the calcium-binding protein S100 β (Grosche et al. 2013; Nishiyama et al.
29 2002). We also overexpressed and imaged membrane-targeted pHluorin and
30 lymphocyte-specific protein tyrosine kinase-green fluorescent protein (Lck-GFP), to
31 understand the expression nano-pattern of fusion proteins which represent a key tool in
32 astroglia-targeted fluorescent imaging (Benediktsson et al. 2005; Endesfelder and
33 Heilemann 2015; Shigetomi et al. 2010). Importantly, we probed these techniques in
34 cultured astroglia (which tend to have a flattened, mainly two-dimensional morphology),
35 in mixed cortical cultures (in which astroglia adopt a more sponge-like morphology) and
36 also in thin brain sections that enable astrocyte structure imaging in three dimensions.
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MATERIALS AND METHODS

Cortical cultures

All animal experiments in this study were carried out in full compliance with the corresponding UK and EU regulations. Cortical dissociated cultures from P0 rats were prepared as follows. We removed brains and isolated cortices on ice in Dissociation Medium (DM; 81.8 mM Na₂SO₄, 30 mM K₂SO₄, 5.8 mM MgCl₂, 0.25 mM CaCl₂, 1 mM HEPES, 20 mM glucose, 1 mM kynureic acid, 0.001% phenol red). Cortices were incubated in DM supplemented with 100 U Papain (Worthington, US-NY) twice for 15 minutes at 37°C; rinsed three times in DM and three times in plating medium (MEM, 10% FBS, 1% Penicillin/Streptomycin, 2mM GlutaMAX [Thermo Fisher Scientific]); triturated in plating medium until no clumps were visible. Cell solution was diluted 1:10 in OptiMEM (Thermo Fisher Scientific) and centrifuged at 1000 rpm for 10 minutes at room temperature. VCell pellet was re-suspended in plating medium, cells counted and plated at a density of 150,000 per 18 mm diameter coverslip (#1.5, scientific laboratory supplies) coated with 1 mg/ml poly-DL-lysine (Sigma) and 25 µg/ml laminin (Sigma). For mixed cortical cultures: three hours after plating medium was exchanged for maintenance medium (Neurobasal-A w/o Phenol Red, 2% B-27 Supplement, 1% Penicillin/Streptomycin, 0.5 mM GlutaMAX, 25 µM β-mercaptoethanol [Thermo Fisher Scientific]). For mixed cortical glial cultures: the next day plating medium was exchanged for glial medium (MEM 0.6% D-glucose, 1% Penicillin/Streptomycin, 10% horse serum 2mM L-glutamine), and cells were fed two to three times a week.

Transfection of cultured cells

Cells were transfected at 7 days *in vitro* (DIV) using Lipofectamine 3000 (Thermo Fisher Scientific) according to manufacturer instructions. Cells were incubated with Lipofectamine/DNA complexes in transfecting medium (MEM, 2% B-27 Supplement, 1 mM pyruvate, 0.5 mM GlutaMAX, 25 µM β-mercaptoethanol [Thermo Fisher Scientific]) for 1 hour at 37°C, 5% CO₂ in humidified incubator. After that cells were put back into their conditioned maintenance medium.

The plasmids that were used in this study were: pZac2.1gfaABC1D_Lck-GFP and pDisplay_SEP. pZac2.1gfaABC1D_Lck-GFP was created with standard cloning techniques based on the following plasmids: pZac2.1gfaABC1D_tdTomato (Addgene #44332, gift from Baljit Khakh (Shigetomi et al. 2013)); pN1_Lck-GCaMP5G (Addgene #34924, gift from Baljit Khakh (Akerboom et al. 2012)); and a plasmid encoding GFP. pDisplay_SEP was created by cloning super-ecliptic pHluorin (SEP) into pDisplay_mSA-eGFP-TM (Addgene # 39863, gift from Sheldon Park (Lippman Bell et al. 2010))

Immunocytochemistry in culture

To perform super-resolution imaging of astrocytes in culture we labelled the cells using the following immunocytochemistry protocol (adapted from (Whelan and Bell 2015)). Pre-extraction was carried out using 0.2% saponin (Generon) in cytoskeleton-stabilization buffer (CSB; 10 mM MES pH 6.0, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 320 mM sucrose [Sigma]; warmed to 37°C) for 1 minute at room temperature. This was followed by: fixation with 3% formaldehyde (TAAB) + 0.1% glutaraldehyde (TAAB) diluted in CSB (warmed to 37°C) for 12-15 minutes at 37°C; washing with phosphate-buffered saline (PBS) twice for 2 and twice for 5 minutes gently rocking at room temperature; quenching with 0.1% NaBH₄ (Sigma) in PBS for 7 minutes gently rocking at room temperature; washing with PBS once for 2 and thrice for 5 minutes gently rocking at room temperature; permeabilization and blocking with blocking buffer (PBS supplemented with 0.1% saponin (PBS-S) and 3% bovine serum albumin (BSA, Sigma)) for 30 minutes gently rocking at room temperature. The samples were next incubated with primary antibodies (

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3 Table 1) in PBS-S supplemented with 1% BSA overnight at 4°C; washed with PBS-S
4 once for 2 and thrice for 10 minutes gently rocking at room temperature; incubated with
5 fluorescently-labelled secondary antibodies (Table 2) diluted in PBS-S for one hour
6 gently rocking at room temperature wrapped with aluminium foil; washed with PBS-S
7 once for 10 minutes gently rocking at room temperature; incubated with fiducial markers
8 (100 nm TetraSpeck microspheres, Thermo Fisher Scientific) diluted 1:500 in PBS for
9 20 minutes at room temperature, and washed with PBS-S once for 10 minutes and with
10 PBS twice for 10 minutes at room temperature. Post fixation was carried out using 4%
11 paraformaldehyde (PFA, Sigma) in PBS for 10 minutes at room temperature followed by
12 washing with PBS thrice for 10 minutes gently rocking at room temperature, and
13 samples were either imaged immediately or stored at 4°C. Seven DIV old mixed cortical
14 glial cell cultures and 14 DIV old mixed cortical cultures were used for
15 immunocytochemistry.
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22 **Immunohistochemistry in brain tissue sections**

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24 To perform super-resolution imaging of astrocytes in brain tissue sections we labelled
25 the cells using the following immunocytochemistry protocol. Deeply anaesthetized rats
26 (Sprague Dawley, 250 g) were perfused with ice-cold 4% PFA in PBS, brains were
27 removed and incubated in 4% PFA in PBS overnight at 4°C, then sectioned using a
28 vibratom (40 µm coronal sections). Sections were kept free-floating in PBS, incubated in
29 0.1% NaBH₄ in PBS for 15 min gently rocking at room temperature, then washed thrice
30 for 5 min with PBS gently rocking at room temperature. Permeabilization and blocking
31 were carried out using blocking buffer (PBS-S supplemented with 3% BSA) for 2 hours
32 gently rocking at room temperature followed by incubation with primary antibodies in
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3 Table 1) overnight gently rocking at 4°C. Samples were washed with PBS-S thrice for
4 10 minutes gently rocking at room temperature, incubated with fluorescently-labelled
5 secondary antibodies (Table 2) diluted in PBS-S for two hours gently rocking at room
6 temperature, washed with PBS-S twice for 10 minutes and with PBS thrice for 5 minutes
7 gently rocking at room temperature. Post fixation was performed using 4% PFA in PBS
8 for 30 minutes gently rocking at room temperature, followed by washing with PBS thrice
9 for 10 minutes gently rocking at room temperature. Sections were incubated in Scale U2
10 buffer (4 M urea, 30% Glycerol and 0.1% Triton X-100 in water; Hama et al. 2011) and
11 stored covered at 4°C until being prepared for imaging.
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Table 1: Primary antibodies used.

ICC = Dilution factor used in immunocytochemistry, IHC = Dilution factor used in immunohistochemistry

Antigen	host	Clone	Supplier	product code	ICC	IHC
GFP	Chicken	polyclonal	Thermo	A10262	1:1000	N/A
GFP	Mouse	monoclonal	Merck	MAB3580	1:1000	N/A
GFAP	Mouse	GA5	Novus	NBP2-29415	1:1000	1:500
Glutamine synthetase	Mouse	monoclonal	abcam	ab64613	1:250	1:200
Glutamine synthetase	Mouse	GS6	Merck	MAB302	1:250	1:200
GFAP	Rabbit	Polyclonal	Novus	NB300-141	1:1000	1:500
S100 β	Rabbit	Polyclonal	Synaptic systems	287 003	1:250	1:200

Table 2: Secondary antibodies used.

ICC = Dilution factor used in immunocytochemistry, IHC = Dilution factor used in immunohistochemistry

Antigen	Feature	Host	Supplier	product code	ICC	IHC
Chicken IgY	Alexa647-conjugated	Goat	Thermo	A21246	1:1000	N/A
Mouse IgG	CF568-conjugated	Donkey	Biotium	20105	1:500	1:200
Rabbit IgG	Alexa647-conjugated	Goat	Thermo	A21245	1:1000	1:500

Super-resolution imaging

Here, we employed the super-resolution imaging technique direct stochastic optical reconstruction microscopy (dSTORM) (Endesfelder and Heilemann 2015; van de Linde et al. 2011). Super-resolution images were recorded with a Vutara 350 commercial microscope (Bruker Corp., Billerica, US-MA) based on the single molecule localization (SML) biplane technology (Juetten et al. 2008; Mlodzianoski et al. 2009). The targets were imaged using 647 nm (for Alexa647) and 561 nm (for CF568) excitation lasers, respectively, and a 405 nm activation laser in a photoswitching buffer containing 100 mM cysteamine and oxygen scavengers (glucose oxidase and catalase) (Metcalf et al. 2013). Images were recorded using a 60x-magnification, 1.2-NA water immersion

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3 objective (Olympus) and a Flash 4.0 sCMOS camera (Hamamatsu) with frame rate at
4 50 Hz. Total number of frames acquired per channel ranged from 5,000 to 20,000
5 frames. Data were analyzed using the Vutara SRX software (version 5.22). Single
6 molecules were identified by their brightness frame by frame after removing the
7 background. Identified particles were then localized in three dimensions by fitting the
8 raw data with a 3D model function, which was obtained from recorded bead data sets.
9 The experimentally achievable image resolution is 20 nm laterally (x and y) and 50 nm
10 axially (z). With our methods we routinely achieve a lateral resolution of 38.2 ± 9.8 nm in
11 cultured cells and 58.02 ± 7.09 nm in tissue sections and an axial resolution of $51.65 \pm$
12 20.61 nm in cultured cells and 73 ± 5.82 nm in tissue sections.
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20 RESULTS

21 Nano localization of GFAP, S100 β , and glutamate synthetase in mixed cortical 22 glial cultures 23 24

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26 First, we sought to image astrocytes in mixed cortical glial cultures. First, we employed
27 the widely used astrocyte marker GFAP (Oberheim et al. 2012). Mixed cortical glial cells
28 were cultured for one week in glial medium prior to fixation and immunostaining. Mouse
29 or rabbit anti-GFAP antibodies were then labelled with secondary antibodies tagged
30 with CF568 or Alexa647, which were used for dSTORM imaging. The images revealed
31 a dotted structure of thin GFAP fibers forming a loose intracellular mesh (Figure 1). We
32 next used a similar approach in an attempt to reveal the nanoscale distribution pattern
33 of two other astroglia marker proteins that represent key cellular cascades and have
34 been used extensively across the astroglial field, glutamine synthetase (Derouiche and
35 Frotscher 1991; Volterra et al. 2014b) and the calcium-binding protein S100 β (Grosche
36 et al. 2013; Nishiyama et al. 2002). Again, mixed cortical glial cells were cultured for one
37 week in glial medium prior to fixation and immunostaining. The images revealed spotty
38 protein clusters, with the macroscopic pattern appearance drastically different from that
39 of GFAP (Figure 2).
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48 Nano localization of GFAP and S100 β in mixed cortical cultures 49

50 Our next step was to visualize the expression patterns of all three marker proteins
51 (GFAP, glutamine synthetase and S100 β) in mixed cortical cultures, two weeks post-
52 plating, using the methods similar to those in the previous section. We found that in the
53 mixed cortical cultures, astrocytes showed a more sponge-like morphology, with several
54 discernable GFAP-positive processes, in contrast to the more spread-out appearance in
55 mixed glial cultures (Figure 3). Also in this culture the cells clearly expressed all the
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3 astrocyte marker proteins under study. The super-resolution imaging revealed thin
4 GFAP fibers that were virtually undetectable in the wide-field images (e.g., Figure 3I,S),
5 in contrast to the dSTORM rendering (Figure 3J,T). Moreover, it appears as though
6 glutamine synthetase and S100 β fill out small astrocyte processes, the observation
7 which has not hitherto been reported using conventional microscopy methods.
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10 11 12 13 **Nanoscopic imaging of the overexpressed pDisplay_SEP and** 14 **pZac2.1gfaABC1D_Lck-GFP in astroglia in mixed cortical cultures** 15 16

17 We next sought to explore the protein expression technique which has commonly been
18 used to visualize fine astrocyte processes *in situ*: one week old mixed cortical cultures
19 were transfected with either pDisplay_SEP or pZac2.1gfaABC1D_Lck-GFP using
20 Lipofectamine 3000 (Thermo Fisher Scientific). This technique has been employed in
21 the past to successfully visualize fine astrocyte processes *in situ* using conventional
22 confocal or two-photon excitation microscopy (Benediktsson et al. 2005; Shigetomi et al.
23 2010). At 14 DIV the transfected cell cultures were used for immunostaining against
24 GFP, to enable visualization the overall morphological outline of the cells under study.
25 dSTORM imaging revealed ultra-thin astrocytic processes (below 100 nm in diameter)
26 that were hardly detectable in the conventional wide-field images (Figure 4E and J).
27 These observations indicated the feasibility of using the overexpression of membrane-
28 targeted fluorescent proteins to resolve the nanostructure of ultrathin astrocytic
29 processes that were beyond the diffraction limit of conventional optical microscopy.
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37 **dSTORM of GFAP expression in organized brain tissue** 38 39

40 Thereafter, we set out to explore nanoscopic localization of GFAP in brain tissue
41 sections. Fixed brains were sectioned (40 μ m coronal sections) and used for
42 immunohistochemistry (Materials and Methods). Once again, we labelled astrocytes
43 using the common astrocytic marker GFAP (Oberheim et al. 2012). Similar to mixed
44 cortical cultures, astrocytes *in situ* showed a sponge-like morphology, with several
45 major GFAP-positive processes (Figure 5A-C). The GFAP fibers appear to be thicker in
46 the proximity of the soma whereas in thin astrocyte processes they could only be
47 identified reliably with super-resolution microscopy (Figure 5E).
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54 **Two-color dSTORM of glutamine synthetase and S100 β in GFAP-labelled** 55 **astroglia in situ** 56 57 58 59 60

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3 Finally, our objective was to obtain the nanoscale expression pattern and co-localization
4 of glutamine synthetase and S100 β in GFAP-expressing cells in brain tissue sections.
5 As in the previous sections, fixed brains were cut into 40 μ m coronal sections used for
6 immunohistochemistry with respect to GFAP (Oberheim et al. 2012) (Figure 6A, K),
7 glutamine synthetase (Derouiche and Frotscher 1991; Volterra et al. 2014b) (Figure 6D)
8 and S100 β (Grosche et al. 2013; Nishiyama et al. 2002) (Figure 6N). dSTORM enabled
9 us to obtain intricate patterns of the three proteins in ultra-thin astroglial processes, the
10 data not attainable with conventional microscopy (Figure 6J,T).
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17 DISCUSSION

19 Nanoscopic astrocyte processes are omnipresent throughout the brain, shielding
20 synapses to a varying degree and providing a platform for neurotransmitter uptake and
21 gliotransmitters release (Bernardinelli et al. 2014b; Haber et al. 2006; Heller and
22 Rusakov 2015; Hirrlinger et al. 2004; Perez-Alvarez et al. 2014; Theodosis et al. 2008).
23 These processes are too small to be reliably detected by conventional microscopy.
24 Therefore, EM has been the only tool used successfully to resolve glial structure on the
25 nanoscale (Bernardinelli et al. 2014c; Lushnikova et al. 2009; Medvedev et al. 2014;
26 Medvedev et al. 2010; Patrushev et al. 2013; Popov et al. 2004; Witcher et al. 2007;
27 Witcher et al. 2010). Here, we documented first attempts to visualize thin astrocytic
28 processes and their nano-organization using the super-resolution microscopy technique
29 dSTORM. Using dSTORM we were able to reconstruct GFAP fibers in astrocytes *in*
30 *vitro* as well as *in situ*. Moreover, we revealed the presence of glutamine synthetase
31 and S100 β in fine astrocytic protrusions, which were undetectable with widefield
32 microscopy in culture and in brain sections. Lastly, we used overexpression of the
33 membrane-targeted fluorescent proteins pHluorin and GFP to illuminate the entire
34 outline of cultured astrocytes. Moreover, dSTORM revealed the presence of thin
35 astrocytic processes that were not visible in conventional microscopy images. Taken
36 together, this study provides evidence for the feasibility of dSTORM as a means to
37 revealing thin astrocytic processes in cultured cells as well as in brain sections. Future
38 experiments involving the analysis of the nano-organization of these processes will
39 shed light on the intricate physiology of these cells protrusions.
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Conflict of Interest Statement

The authors declare no conflict of interest.

Author contribution

Conception and design: JH and DR

Acquisition and analysis: JH, PM and KS

Drafting and revision of work: JH, PM and DR

Manuscript writing: JH and DR

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REFERENCES

- 1
2
3
4
5
6
7
8
9 Agulhon C, Petravicz J, McMullen AB, Sweger EJ, Minton SK, Taves SR, Casper KB,
10 Fiocco TA, McCarthy KD. 2008. What is the role of astrocyte calcium in
11 neurophysiology? *Neuron* 59(6):932-946.
- 12
13 Akerboom J, Chen TW, Wardill TJ, Tian L, Marvin JS, Mutlu S, Calderon NC, Esposti F,
14 Borghuis BG, Sun XR, Gordus A, Orger MB, Portugues R, Engert F, Macklin JJ,
15 Filosa A, Aggarwal A, Kerr RA, Takagi R, Kracun S, Shigetomi E, Khakh BS,
16 Baier H, Lagnado L, Wang SS, Bargmann CI, Kimmel BE, Jayaraman V,
17 Svoboda K, Kim DS, Schreier ER, Looger LL. 2012. Optimization of a GCaMP
18 calcium indicator for neural activity imaging. *J Neurosci* 32(40):13819-13840.
- 19
20 Araque A, Carmignoto G, Haydon PG, Oliet SH, Robitaille R, Volterra A. 2014.
21 Gliotransmitters travel in time and space. *Neuron* 81(4):728-739.
- 22
23 Bates M, Huang B, Dempsey GT, Zhuang X. 2007. Multicolor super-resolution imaging
24 with photo-switchable fluorescent probes. *Science* 317(5845):1749-1753.
- 25
26 Bazargani N, Attwell D. 2016. Astrocyte calcium signaling: the third wave. *Nat Neurosci*
27 19(2):182-189.
- 28
29 Benediktsson AM, Schachtele SJ, Green SH, Dailey ME. 2005. Ballistic labeling and
30 dynamic imaging of astrocytes in organotypic hippocampal slice cultures. *Journal*
31 *of neuroscience methods* 141(1):41-53.
- 32
33 Bennett MV, Garre JM, Orellana JA, Bukauskas FF, Nedergaard M, Saez JC. 2012.
34 Connexin and pannexin hemichannels in inflammatory responses of glia and
35 neurons. *Brain Res* 1487:3-15.
- 36
37 Bergles DE, Diamond JS, Jahr CE. 1999. Clearance of glutamate inside the synapse
38 and beyond. *Curr Opin Neurobiol* 9(3):293-298.
- 39
40 Bernardinelli Y, Muller D, Nikonenko I. 2014a. Astrocyte-synapse structural plasticity.
41 *Neural plasticity* 2014:232105.
- 42
43 Bernardinelli Y, Randall J, Janett E, Nikonenko I, Konig S, Jones EV, Flores CE, Murai
44 KK, Bochet CG, Holtmaat A, Muller D. 2014b. Activity-dependent structural
45 plasticity of perisynaptic astrocytic domains promotes excitatory synapse
46 stability. *Current biology : CB* 24(15):1679-1688.
- 47
48 Bernardinelli Y, Randall J, Janett E, Nikonenko I, Konig S, Jones EV, Flores CE, Murai
49 KK, Bochet CG, Holtmaat A, Muller D. 2014c. Activity-dependent structural
50 plasticity of perisynaptic astrocytic domains promotes excitatory synapse
51 stability. *Curr Biol* 24(15):1679-1688.
- 52
53 Betzig E, Patterson GH, Sougrat R, Lindwasser OW, Olenych S, Bonifacino JS,
54 Davidson MW, Lippincott-Schwartz J, Hess HF. 2006. Imaging intracellular
55 fluorescent proteins at nanometer resolution. *Science* 313(5793):1642-1645.
- 56
57 Danbolt NC. 2001. Glutamate uptake. *Progr Neurobiol* 65:1-105.
- 58
59 Derouiche A, Frotscher M. 1991. Astroglial processes around identified glutamatergic
60 synapses contain glutamine synthetase: evidence for transmitter degradation.
Brain research 552(2):346-350.
- Dityatev A, Rusakov DA. 2011. Molecular signals of plasticity at the tetrapartite
synapse. *Curr Opin Neurobiol* 21(2):353-359.

- 1
2
3 Endesfelder U, Heilemann M. 2015. Direct stochastic optical reconstruction microscopy
4 (dSTORM). *Methods in molecular biology* 1251:263-276.
- 5
6 Grosche A, Grosche J, Tackenberg M, Scheller D, Gerstner G, Gumprecht A, Pannicke
7 T, Hirrlinger PG, Wilhelmsson U, Huttmann K, Hartig W, Steinhauser C, Pekny
8 M, Reichenbach A. 2013. Versatile and simple approach to determine astrocyte
9 territories in mouse neocortex and hippocampus. *PloS one* 8(7):e69143.
- 10
11 Haber M, Zhou L, Murai KK. 2006. Cooperative astrocyte and dendritic spine dynamics
12 at hippocampal excitatory synapses. *The Journal of neuroscience : the official
13 journal of the Society for Neuroscience* 26(35):8881-8891.
- 14
15 Halassa MM, Haydon PG. 2010. Integrated brain circuits: astrocytic networks modulate
16 neuronal activity and behavior. *Annu Rev Physiol* 72:335-355.
- 17
18 Hama H, Kurokawa H, Kawano H, Ando R, Shimogori T, Noda H, Fukami K, Sakaue-
19 Sawano A, Miyawaki A. 2011. Scale: a chemical approach for fluorescence
20 imaging and reconstruction of transparent mouse brain. *Nature neuroscience*
21 14(11):1481-1488.
- 22
23 Haydon PG. 2001. GLIA: listening and talking to the synapse. *Nat Rev Neurosci*
24 2(3):185-193.
- 25
26 Heller JP, Rusakov DA. 2015. Morphological plasticity of astroglia: Understanding
27 synaptic microenvironment. *Glia*.
- 28
29 Hirrlinger J, Hulsman S, Kirchhoff F. 2004. Astroglial processes show spontaneous
30 motility at active synaptic terminals in situ. *The European journal of neuroscience*
31 20(8):2235-2239.
- 32
33 Huang B, Wang W, Bates M, Zhuang X. 2008. Three-dimensional super-resolution
34 imaging by stochastic optical reconstruction microscopy. *Science* 319(5864):810-
35 813.
- 36
37 Juette MF, Gould TJ, Lessard MD, Mlodzianoski MJ, Nagpure BS, Bennett BT, Hess
38 ST, Bewersdorf J. 2008. Three-dimensional sub-100 nm resolution fluorescence
39 microscopy of thick samples. *Nat Methods* 5(6):527-529.
- 40
41 Khakh BS, Sofroniew MV. 2015. Diversity of astrocyte functions and phenotypes in
42 neural circuits. *Nat Neurosci* 18(7):942-952.
- 43
44 Klar TA, Jakobs S, Dyba M, Egnér A, Hell SW. 2000. Fluorescence microscopy with
45 diffraction resolution barrier broken by stimulated emission. *Proceedings of the
46 National Academy of Sciences of the United States of America* 97(15):8206-
47 8210.
- 48
49 Lippman Bell JJ, Lordkipanidze T, Cobb N, Dunaevsky A. 2010. Bergmann glial
50 ensheathment of dendritic spines regulates synapse number without affecting
51 spine motility. *Neuron glia biology* 6(3):193-200.
- 52
53 Lushnikova I, Skibo G, Muller D, Nikonenko I. 2009. Synaptic potentiation induces
54 increased glial coverage of excitatory synapses in CA1 hippocampus.
55 *Hippocampus* 19(8):753-762.
- 56
57 Matyash V, Kettenmann H. 2010. Heterogeneity in astrocyte morphology and
58 physiology. *Brain Res Rev* 63(1-2):2-10.
- 59
60 Medvedev N, Popov V, Henneberger C, Kraev I, Rusakov DA, Stewart MG. 2014. Glia
selectively approach synapses on thin dendritic spines. *Philos Trans R Soc Lond
B Biol Sci* 369(1654).

- 1
2
3 Medvedev NI, Popov VI, Rodriguez Arellano JJ, Dallerac G, Davies HA, Gabbott PL,
4 Laroche S, Kraev IV, Doyere V, Stewart MG. 2010. The N-methyl-D-aspartate
5 receptor antagonist CPP alters synapse and spine structure and impairs long-
6 term potentiation and long-term depression induced morphological plasticity in
7 dentate gyrus of the awake rat. *Neurosci* 165(4):1170-1181.
- 8
9 Metcalf DJ, Edwards R, Kumarswami N, Knight AE. 2013. Test samples for optimizing
10 STORM super-resolution microscopy. *J Vis Exp*(79).
- 11 Mlodzianoski MJ, Juette MF, Beane GL, Bewersdorf J. 2009. Experimental
12 characterization of 3D localization techniques for particle-tracking and super-
13 resolution microscopy. *Opt Express* 17(10):8264-8277.
- 14 Nishiyama H, Knopfel T, Endo S, Itohara S. 2002. Glial protein S100B modulates long-
15 term neuronal synaptic plasticity. *Proceedings of the National Academy of*
16 *Sciences of the United States of America* 99(6):4037-4042.
- 17
18 Oberheim NA, Goldman SA, Nedergaard M. 2012. Heterogeneity of astrocytic form and
19 function. *Methods in molecular biology* 814:23-45.
- 20 Panatier A, Arizono M, Nagerl UV. 2014. Dissecting tripartite synapses with STED
21 microscopy. *Philosophical transactions of the Royal Society of London Series B,*
22 *Biological sciences* 369(1654):20130597.
- 23 Patrushev I, Gavrillov N, Turlapov V, Semyanov A. 2013. Subcellular location of
24 astrocytic calcium stores favors extrasynaptic neuron-astrocyte communication.
25 *Cell Calcium* 54(5):343-349.
- 26
27 Perez-Alvarez A, Navarrete M, Covelo A, Martin ED, Araque A. 2014. Structural and
28 functional plasticity of astrocyte processes and dendritic spine interactions. *The*
29 *Journal of neuroscience : the official journal of the Society for Neuroscience*
30 34(38):12738-12744.
- 31
32 Popov VI, Davies HA, Rogachevsky VV, Patrushev IV, Errington ML, Gabbot PLA, Bliss
33 TVP, Stewart MG. 2004. Remodelling of synaptic morphology but unchanged
34 synaptic density during late phase long-term potentiation (LTP): A serial section
35 electron micrograph study in the dentate gyrus in the anaesthetised rat. *Neurosci*
36 128(2):251-262.
- 37
38 Porter JT, McCarthy KD. 1997. Astrocytic neurotransmitter receptors in situ and in vivo.
39 *Prog Neurobiol* 51(4):439-455.
- 40
41 Rossi A, Moritz TJ, Ratelade J, Verkman AS. 2012. Super-resolution imaging of
42 aquaporin-4 orthogonal arrays of particles in cell membranes. *J Cell Sci* 125(Pt
43 18):4405-4412.
- 44
45 Rusakov DA. 2015. Disentangling calcium-driven astrocyte physiology. *Nature Rev*
46 *Neurosci* 16(4):226-233.
- 47
48 Rusakov DA, Bard L, Stewart MG, Henneberger C. 2014. Diversity of astroglial
49 functions alludes to subcellular specialisation. *Trends Neurosci* 37(4):228-242.
- 50 Seifert G, Carmignoto G, Steinhauser C. 2010. Astrocyte dysfunction in epilepsy. *Brain*
51 *Res Rev* 63(1-2):212-221.
- 52 Shigetomi E, Bushong EA, Haustein MD, Tong X, Jackson-Weaver O, Kracun S, Xu J,
53 Sofroniew MV, Ellisman MH, Khakh BS. 2013. Imaging calcium microdomains
54 within entire astrocyte territories and endfeet with GCaMPs expressed using
55 adeno-associated viruses. *J Gen Physiol* 141(5):633-647.
- 56
57
58
59
60

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2
3 Shigetomi E, Kracun S, Khakh BS. 2010. Monitoring astrocyte calcium microdomains
4 with improved membrane targeted GCaMP reporters. *Neuron glia biology*
5 6(3):183-191.
6
7 Smith AJ, Verkman AS. 2015. Superresolution Imaging of Aquaporin-4 Cluster Size in
8 Antibody-Stained Paraffin Brain Sections. *Biophysical journal* 109(12):2511-
9 2522.
10
11 Theodosis DT, Poulain DA, Oliet SH. 2008. Activity-dependent structural and functional
12 plasticity of astrocyte-neuron interactions. *Physiological reviews* 88(3):983-1008.
13
14 van de Linde S, Loschberger A, Klein T, Heidbreder M, Wolter S, Heilemann M, Sauer
15 M. 2011. Direct stochastic optical reconstruction microscopy with standard
16 fluorescent probes. *Nat Protoc* 6(7):991-1009.
17
18 Verkhratsky A, Sofroniew MV, Messing A, deLanerolle NC, Rempe D, Rodriguez JJ,
19 Nedergaard M. 2012. Neurological diseases as primary gliopathies: a
20 reassessment of neurocentrism. *ASN Neuro* 4(3).
21
22 Volterra A, Liaudet N, Savtchouk I. 2014a. Astrocyte Ca²⁺ signalling: an unexpected
23 complexity. *Nat Rev Neurosci* 15(5):327-335.
24
25 Volterra A, Liaudet N, Savtchouk I. 2014b. Astrocyte Ca(2)(+) signalling: an unexpected
26 complexity. *Nature reviews Neuroscience* 15(5):327-335.
27
28 Volterra A, Meldolesi J. 2005. Astrocytes, from brain glue to communication elements:
29 the revolution continues. *Nature Rev Neurosci* 6(8):626-640.
30
31 Whelan DR, Bell TD. 2015. Image artifacts in single molecule localization microscopy:
32 why optimization of sample preparation protocols matters. *Sci Rep* 5:7924.
33
34 Witcher MR, Kirov SA, Harris KM. 2007. Plasticity of perisynaptic astroglia during
35 synaptogenesis in the mature rat hippocampus. *Glia* 55(1):13-23.
36
37 Witcher MR, Park YD, Lee MR, Sharma S, Harris KM, Kirov SA. 2010. Three-
38 dimensional relationships between perisynaptic astroglia and human
39 hippocampal synapses. *Glia* 58(5):572-587.
40
41 Zorec R, Araque A, Carmignoto G, Haydon PG, Verkhratsky A, Parpura V. 2012.
42 Astroglial excitability and gliotransmission: an appraisal of Ca²⁺ as a signalling
43 route. *ASN Neuro* 4(2).
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FIGURE LEGEND

Figure 1: dSTORM imaging of GFAP in astrocytes in mixed cortical glial cultures.

A: Wide-field image of astrocytes in mixed glial cultures expressing GFAP.

B: Area shown by rectangle in **A** at higher magnification, wide-field fluorescence mode.

C: Area as in **B** shown as a dSTORM image; GFAP fibers appear super-resolved, with single molecules making up the intermediate filament made discernible; blue clusters, fiducial markers used for drift correction. False color scale, focal depth.

D: Area shown by rectangle in **B** at higher magnification, wide-field fluorescence mode.

E: Area as in **B** shown as a dSTORM image; more detail and numerous individual GFAP fibers are visible at higher magnification.

F-J: Another example, notations and labelling are the same as in **A-E**.

Scale bars: A-C and F-H, 5 μm ; D-E and I-J, 1 μm .

Figure 2: Nanoscopy of astrocytic marker proteins glutamine synthetase and S100 β in mixed cortical glial cultures.

A: Wide-field image of astrocytes in mixed glial cultures expressing GFAP.

B: Area shown by rectangle in **A** at higher magnification, wide-field fluorescence mode.

C: A dSTORM image of area shown on **B**; individual molecules constituting the intermediate filament are visualized.

D: Wide-field image of glutamine synthetase (GS) in the same field of view as in **A**.

E: Area shown by rectangle in **D** at higher magnification, wide-field fluorescence mode.

F: A dSTORM image of area shown in **E**. Locations of individual glutamine synthetase molecules can be seen.

G: Merged image of **B** and **E**, with GFAP shown in green and GS in magenta, wide-field fluorescence mode.

H: Merged image of **F** and **H**, with GFAP shown in green and GS in magenta, dSTORM.

I-J: Areas shown by rectangles in **G** and **H**, respectively. Individual GFAP fibers, as well as clusters of glutamine synthetase, are visible at higher magnification in the dSTORM image (**J**) while appearing undiscernible in wide-field mode (**I**).

K-T: A series of images, wide-field and dSTORM, illustrating expression pattern and nanoscopic co-localization of GFAP and S100 β in mixed cortical glial cultures. GFAP is shown in green and S100 β in magenta. Other notations are the same as in **A-J**.

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3 Scale bars: A-H and K-R, 5 μm ; I-J and S-T, 1 μm .
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7 **Figure 3: Comparison of GFAP, glutamine synthetase and S100 β expression in**
8 **mixed cortical cultures.**
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10 **A-T:** Wide-field and dSTORM images of astrocyte compartments in mixed cortical
11 cultures, with the same notation and labelling as in Figure 2.
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13 Scale bars: A-H and K-R, 5 μm ; I-J and S-T, 1 μm .
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16 **Figure 4: Overexpression of membrane-targeted pHluorin or Ick-GFP enables**
17 **visualization of nanoscopic astrocytic processes.**
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19 **A:** Wide-field image highlighting the presence of pHluorin in transfected astroglia.
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21 **B:** Area shown by a rectangle in **A** at higher magnification, wide-field fluorescence
22 mode.
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24 **C:** A dSTORM image of the area shown in **B**. Super-resolution image reveals the
25 presence of distinct astrocytic domains that were unresolved with wide-field microscopy.
26

27 **D and E:** Areas shown by rectangles in **B** and **C**, respectively, at higher magnification in
28 dSTORM mode. Image in E compared to the blown up wide-field images (D
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30 **F-J:** Wide-field and dSTORM images of an astroglia transfected with and illuminated by
31 Ick-GFP; other notation and labelling are the same as in **A-E**.
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33 Scale bars: A-C and F-H, 5 μm ; D-E and I-J, 1 μm .
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36 **Figure 5: dSTORM imaging of GFAP in brain tissue sections.**
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38 **A:** An example of a wide-field image of GFAP-labelled astrocytes in brain sections.
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40 **B:** Area shown by a rectangle in **A** at higher magnification, wide-field fluorescence
41 mode.
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43 **C:** A dSTORM image of area shown in **B**; GFAP fibers are resolved at the nanoscale,
44 with individual discernable intermediate filaments.
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46 **D:** Area shown by a rectangle in **B** at higher magnification, wide-field fluorescence
47 mode.
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49 **E:** Area shown by a rectangle in **C** at higher magnification, dSTORM.
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51 Scale bars: A-C, 5 μm ; D-E, 1 μm .
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54 **Figure 6: Nanoscopy of glutamine synthetase and S100 β in GFAP-expressing**
55 **cells in brain tissue sections.**
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A-T: Wide-field and dSTORM images of astrocyte compartments in brain tissue sections. Notation and labelling are the same as in Figure 2 and 3.
Scale bars: A-H and K-R, 5 μm ; I-J and S-T, 1 μm .

For Peer Review

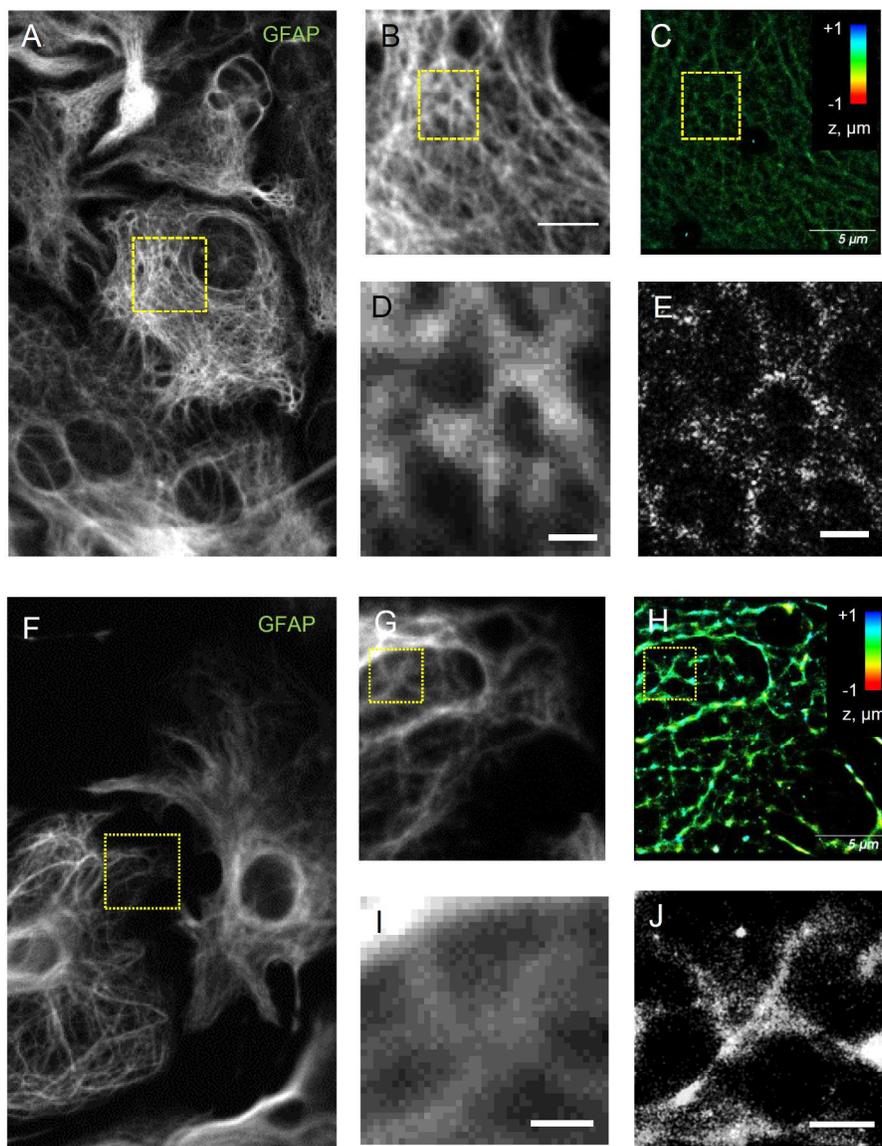


Figure 1

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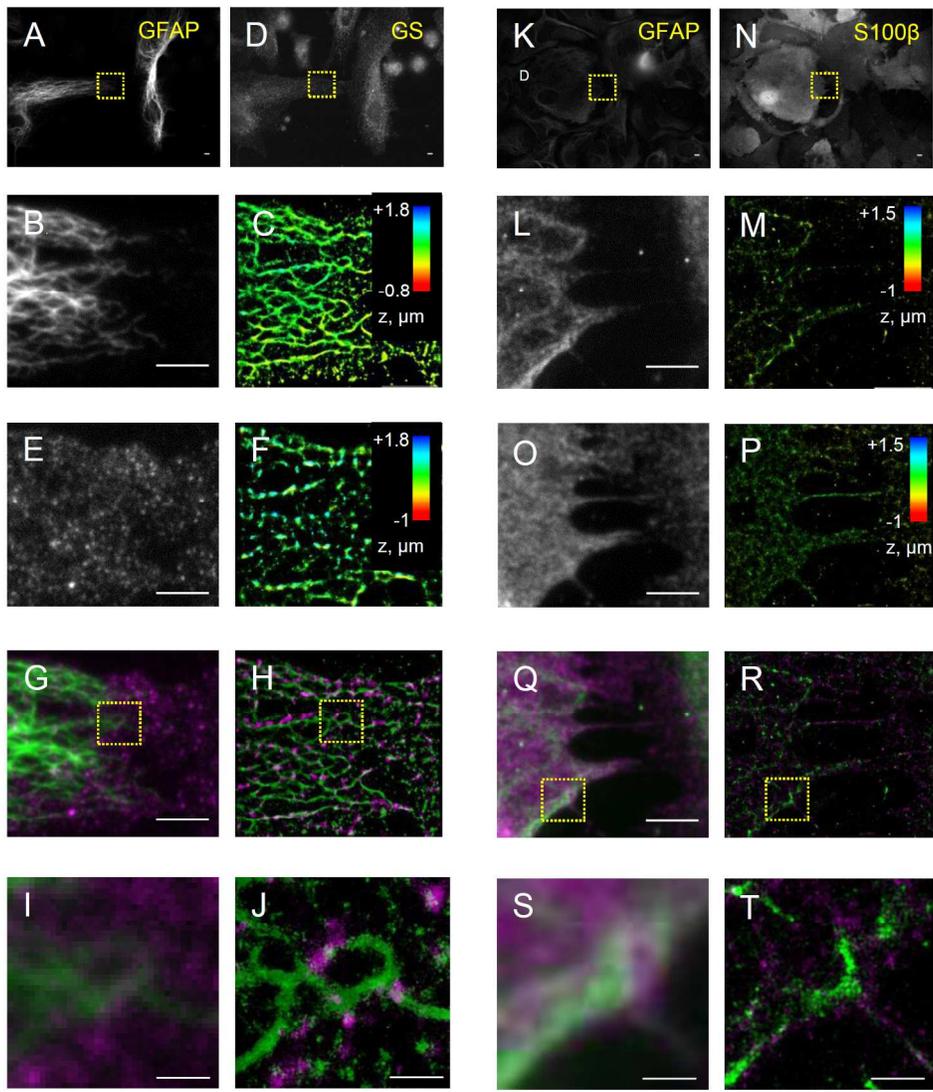


Figure 2

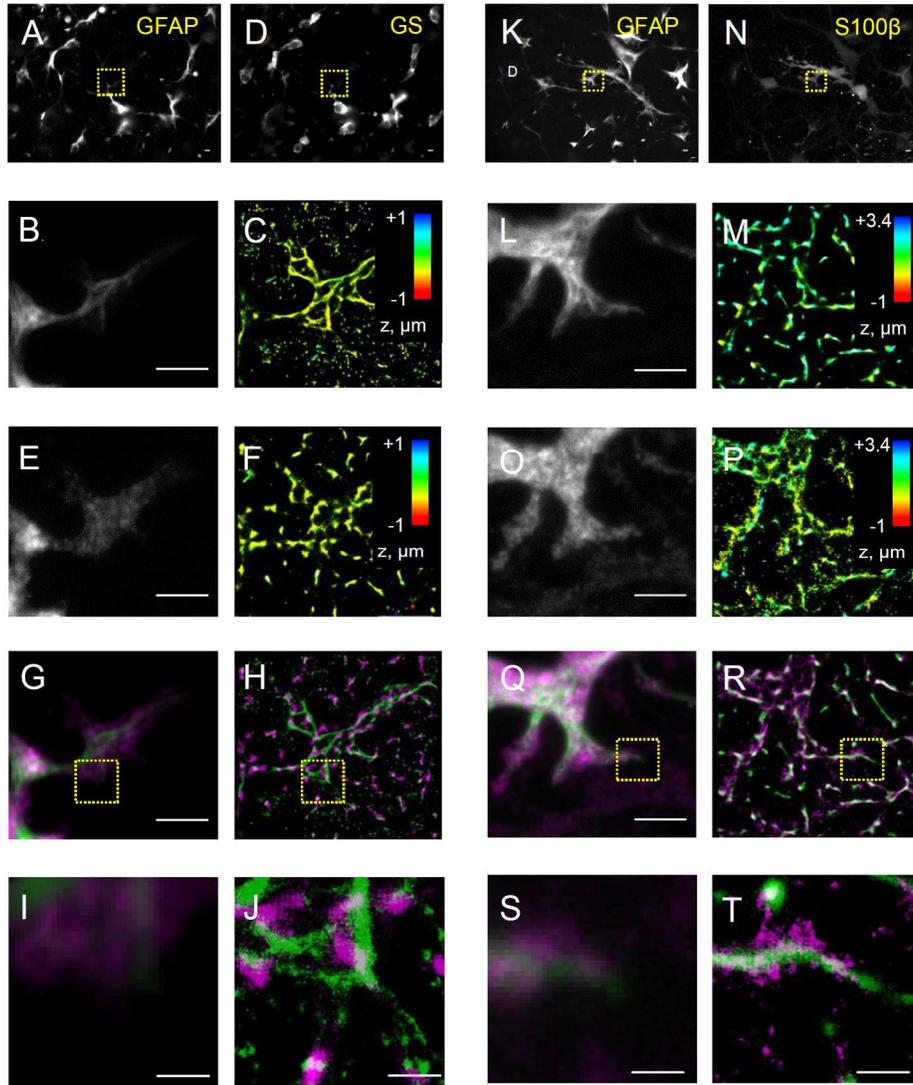


Figure 3

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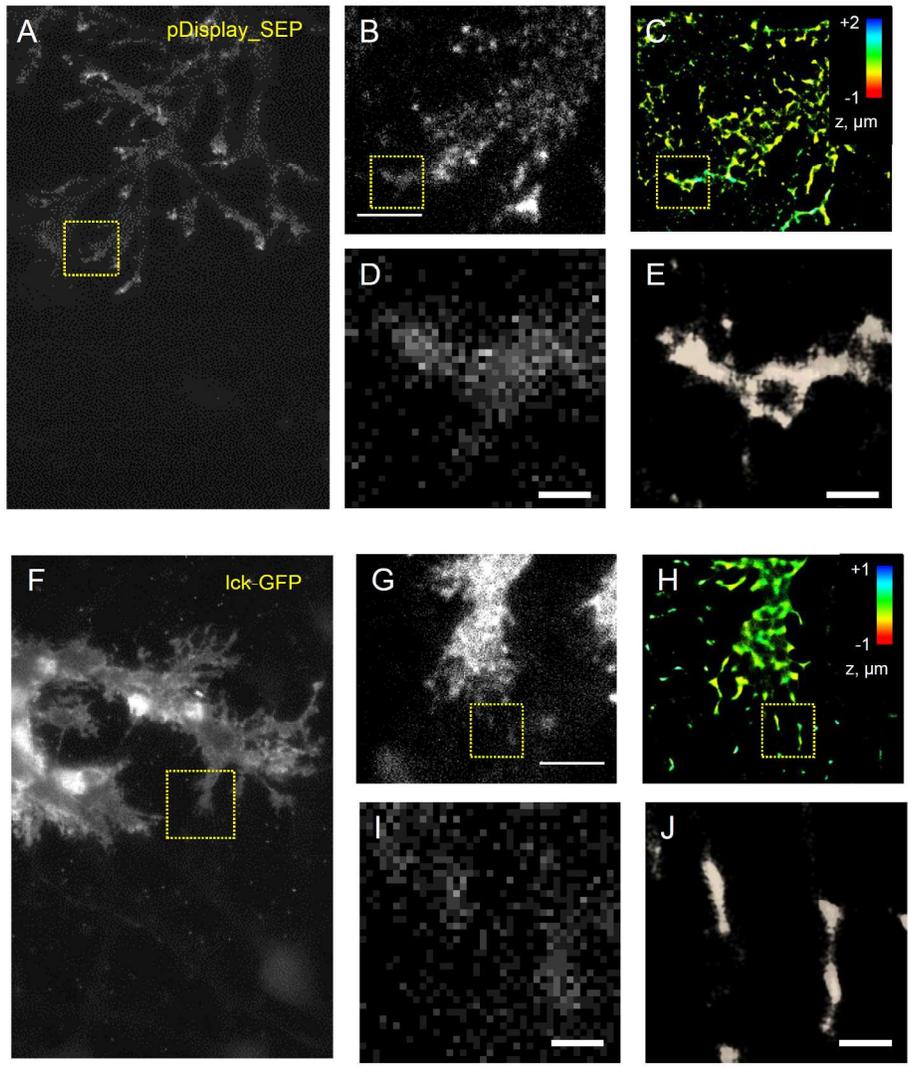
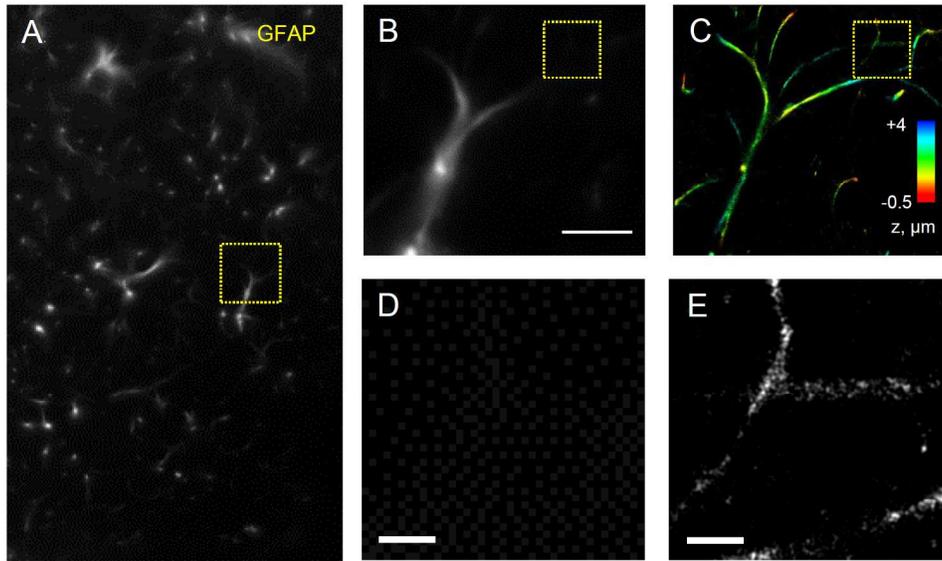


Figure 4

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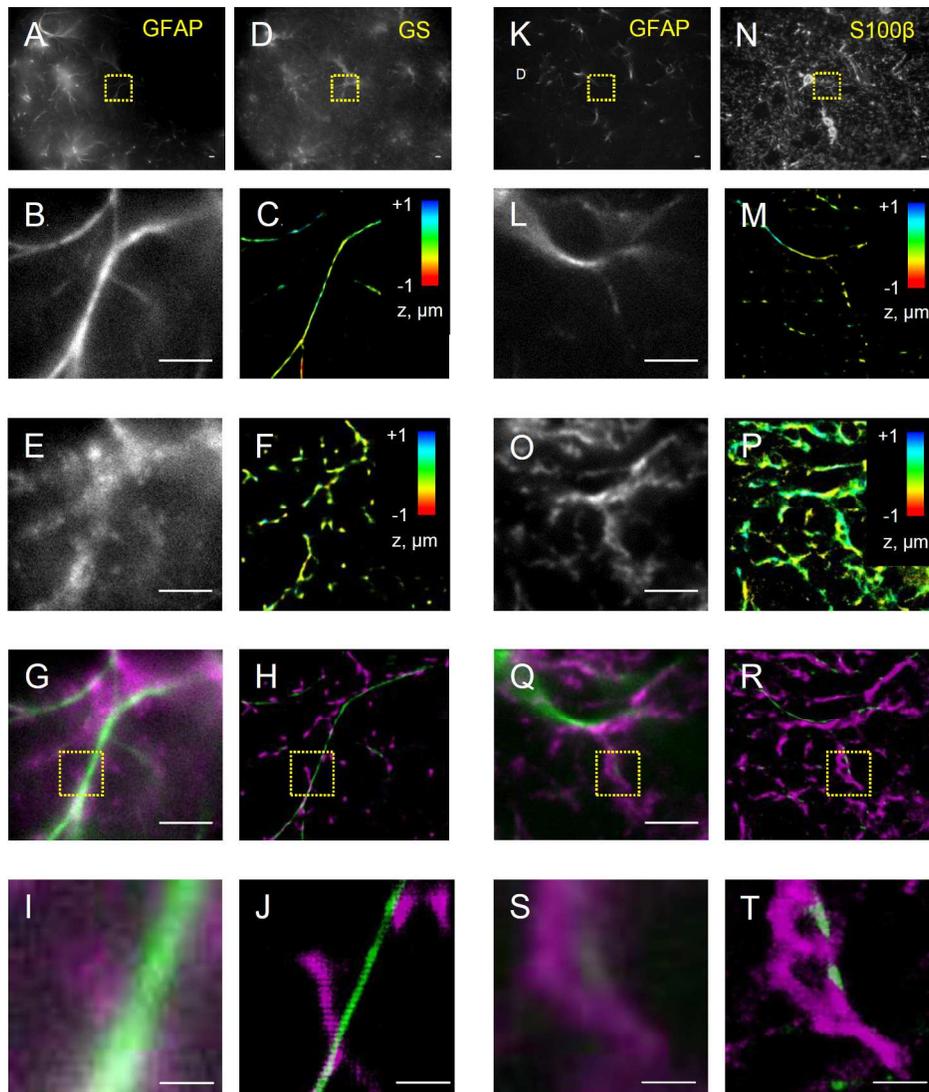


Figure 6