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NEUROSCIENCES
NOVEL-RESULT

New tools for the visualization of glial fibrillary acidic protein in living cells

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#Equal contribution

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Abstract
The glial fibrillary acidic protein (GFAP) is an intermediate filament widely used to identify and label astroglial cells, a very abundant and relevant glial cell type in the central nervous system. A major hurdle in studying its behavior and function arises from the fact that GFAP does not tolerate well the addition of protein tags to its termini. Here, we tagged human GFAP (hGFAP) with an enhanced green fluorescent protein (EGFP) for the first time, and substituted a previously reported EGFP tag on mouse GFAP (mGFAP) by a more versatile Halo Tag. Both versions of tagged GFAP were able to incorporate into the normal GFAP filamentous network in glioma cells, and Alexander disease-related mutations or pharmacological disruption of microtubules and actin filaments interfered with GFAP dynamics. These new tools could provide new fruitful venues for the study of GFAP oligomerization, aggregation and dynamics in living cells.

Key words: glial fibrillary acidic protein; transposase; Halo Tag; single-molecule; SRRF

Introduction

The glial fibrillary acidic protein (GFAP) is an intermediate filament widely known as a molecular marker for astroglia (Middeldorp & Hol, 2011). GFAP expression is strikingly enhanced in conditions of stress, inflammation or injury (Anderson, Ao, & Sofroniew, 2014; Li *et al.*, 2019; Middeldorp & Hol, 2011), and mutations in this filament are associated to Alexander disease (Messing, 2018). However, the structure and function of this filament remain mostly unknown. This is due, at least in part, to the intolerance of GFAP to tags at the N- or C-terminus. Most GFAP studies were restricted to fixed cells and immunocytochemistry (Hsiao *et al.*, 2005; Perng *et al.*, 2008), and early attempts to tag human GFAP (hGFAP) had mixed success, as cells often showed GFAP aggregation or poor filament formation (Bachetti *et al.*, 2008; Perng *et al.*, 2008; Tulyeu *et al.*, 2019). Very recently, mouse GFAP (mGFAP) was successfully tagged with EGFP by introducing a linker between the filament and the fluorescent protein (Mignot *et al.*, 2007). Although mGFAP shares 95% homology to hGFAP (Anderson *et al.*, 2014; Middeldorp & Hol, 2011), there are relevant differences that could determine different mechanisms of regulation and function.

Objective

Here, we developed new tools for the study of GFAP behavior and function in living cells. First, we created an EGFP-tagged version of hGFAP where the tag is within the GFAP sequence, and not at its N- or C-terminus, in order to avoid the issues related to terminal GFAP tags (Bachetti *et al.*, 2008; Perng *et al.*, 2008; Tulyeu *et al.*, 2019). Secondly, we used a previously reported mGFAP construct tagged with EGFP at the C-terminus (Mignot *et al.*, 2007) to insert a Halo Tag in substitution of EGFP. The Halo tag is far more versatile than EGFP (England, Luo, & Cai, 2015) and will allow super-resolution, single-molecule and protein–protein interaction studies on GFAP.

Methods

A commercial transposase was used to insert a kanamycin resistance gene and EGFP into the human GFAP sequence in a random manner (dx.doi.org/10.17504/protocols.io.77fhrjn). The final selected construct had EGFP inserted after amino acid 183 of the GFAP sequence, but the transposase reaction produced an unavoidable repetition of amino acids 181–183 right after the EGFP sequence, as previously described (Sheridan *et al.*, 2002). EGFP was substituted by a Halo tag in the pEGFP-N3-mGFAP plasmid (dx.doi.org/10.17504/protocols.io.77fhrjn). Single R236H or R239C Alexander disease-related mutations were inserted by site-directed mutagenesis into our mGFAP or hGFAP constructs, respectively (dx.doi.org/10.17504/protocols.io.77fhrjn). Human U251 cells and rat C6 glioma cells were maintained, transiently transfected with the corresponding plasmids and treated as described in dx.doi.org/10.17504/protocols.io.77ehrje. Imaging and image analysis were carried out as described in dx.doi.org/10.17504/protocols.io.77ghrjw. Protein extraction and western blotting was carried out as described previously (Herrera *et al.*, 2009). Statistical analysis and graphical representation of data were performed using Sigmaplot software (Systat Software, Inc., San Jose, CA, USA). Sample data are represented as mean ± standard deviation of at least 3 independent experiments. Statistical significance was evaluated by means of a one-way ANOVA followed by a Tukey's test. Results were considered significant when $p < 0.05$.

Results

Both EGFP-hGFAP and mGFAP-Halo constructs formed normal filaments in U251 glioma cells, but the mGFAP-Halo construct failed to do so in rat C6 glioma cells (Fig. 1A). They were suitable for obtaining super-resolution-like pictures by means of super-resolution radial fluctuations (SRRF) (Fig. 1B). None of them formed normal fibers in HEK293 cells (Fig. 1D). The site of insertion of EGFP in hGFAP was not suitable for hosting a Halo tag or bimolecular fluorescence complementation tags (Fig. 1C). While the Alexander disease-related mutation R239C induced aggregation of hGFAP in 48% of cells, the equivalent R236H mutant mGFAP showed disorganization of fibers with signs of aggregation in only 14% of cells (Fig. 2A-C). The expression levels of mutant GFAP were similar to their wild type counterparts (Fig. 2E). However, we cannot rule out the possibility that mGFAP-Halo constructs are expressed at lower overall levels than hGFAP-EGFP constructs, which could explain a lower level of aggregation in mutant mGFAP-Halo. The anti-amyloidogenic curcumin derivative CNB-001 (10 μM)(Liu, Dargusch, Maher, & Schubert, 2008) partially prevented hGFAP aggregation (Fig. 2D). Single-molecule analysis of mGFAP-Halo dynamics confirmed that the R236H mutation produced a higher proportion of free-moving GFAP molecules, and indicated that pharmacological interference with microtubules or actin filaments significantly disrupted mGFAP dynamics (Fig. 3).

Discussion

Our tagged hGFAP and mGFAP constructs behaved mostly as expected for endogenous GFAP when they were expressed in living glioma cells and challenged with previously known genetic and pharmacological modifiers of GFAP fibrillization. The only anomalies detected are the lack of GFAP aggregates

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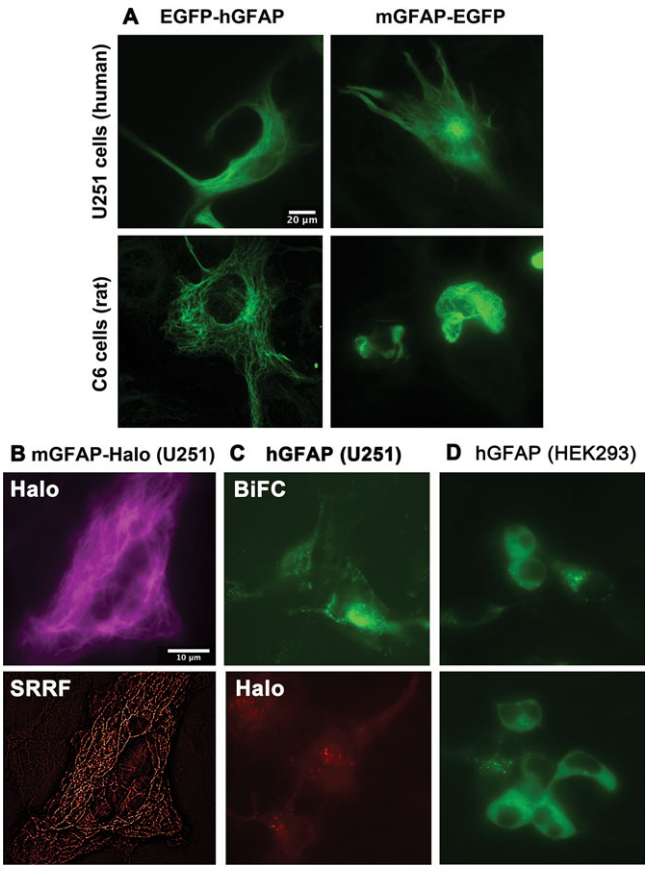

Figure 1. Tagged versions of human and mouse GFAP form normal fibers in glioblastoma cells. Human U251 or rat C6 glioblastoma cells were transiently transfected with different human or mouse GFAP constructs, and imaged 24 h later. A, When transfected into U251 human glioblastoma cells EGFP-hGFAP and mGFAP-EGFP exhibited a normal GFAP filamentous network. However, when the same constructs were transfected into C6 rat glioma cells, only EGFP-hGFAP formed a regular filamentous network. B, mGFAP-Halo constructs (incubated with the JF549 Halo ligand, 100 nM) also produced normal filaments only in U251 cells. Widefield images of the mGFAP-Halo construct were further analyzed using the ImageJ software with the NanoJ SRRF plug-in to obtain a more defined image of the intermediate filament network. C, Our attempts to substitute EGFP for bimolecular fluorescence complementation (BiFC) tags Venus 1 (amino acids 1–157) and Venus 2 (amino acids 158–238) or Halo Tag were unsuccessful. These are representative images of U251 cells transfected with these constructs, where residual fluorescence can be observed but has no recognizable pattern (*i.e.* filaments, bundles or aggregates). D, Transfection of HEK293 cells with the EGFP-hGFAP construct produced either homogenous fluorescence or aggregates, but no filamentous network.

in the R236H mGFAP-Halo mutant and its inability to form fibers in rat glioma cells. These are facts that we cannot currently explain and in which the performance of the EGFP-hGFAP version was superior. Interestingly, the only functional site of EGFP insertion in hGFAP is located in a region (amino acids 183–184) that shows very little frequency of mutations related to Alexander disease (Messing, 2018).

Conclusions

We reported a successful attempt to EGFP-tag hGFAP and a new version of mGFAP tagged with Halo. The EGFP-hGFAP construct is adaptable to different species and behaves as expected when it is mutated, but it is not suitable for single molecule analysis (although it can be used for super-resolution imaging in combination with the SRRF plug-in (Gustafsson *et al.*, 2016). On the other hand, the mGFAP-Halo

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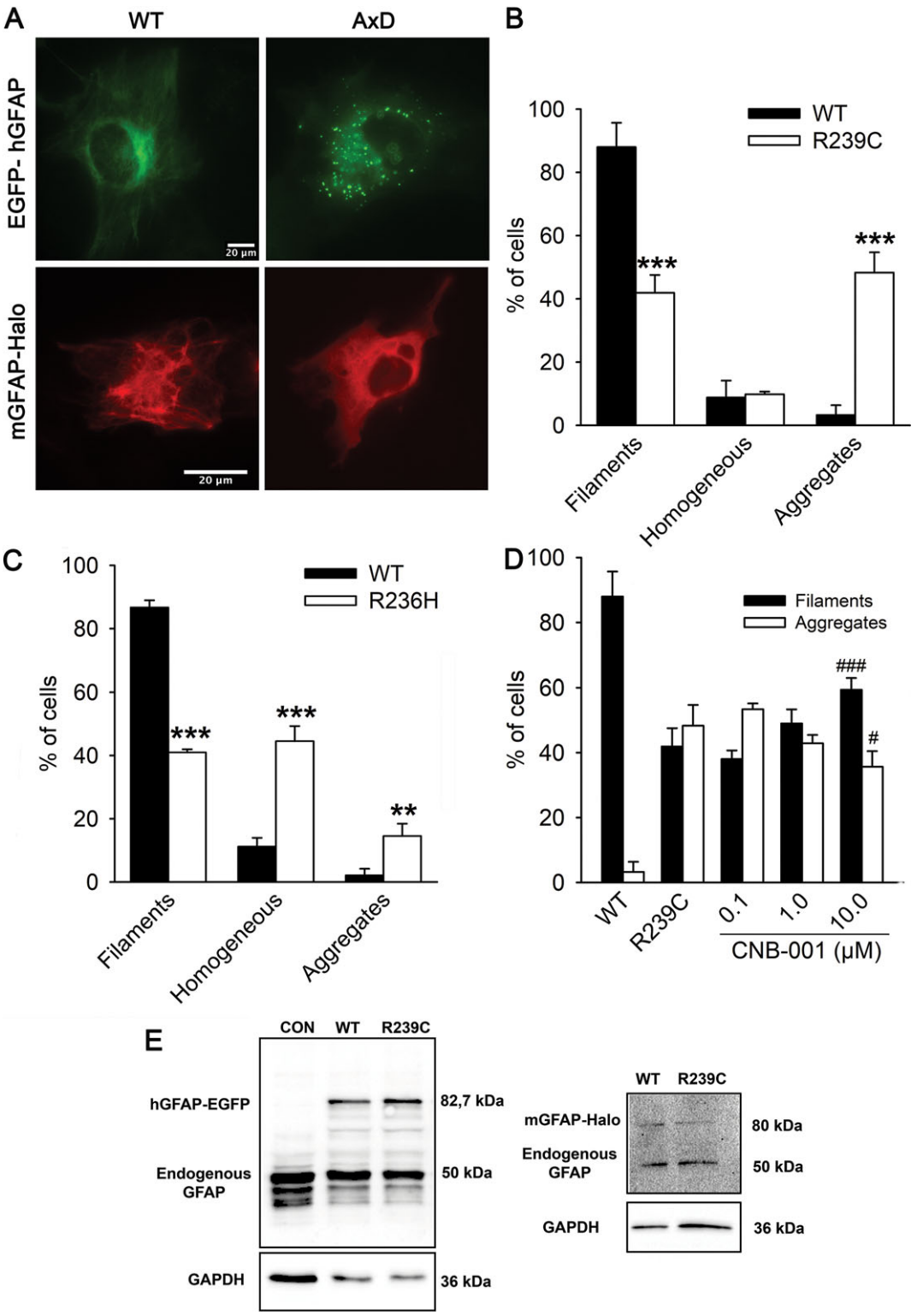

Figure 2. Alexander disease-related mutations cause filament disorganization of GFAP. A, U251 cells were transiently transfected with either wild type (WT) or Alexander disease (AxD)-related versions of EGFP-hGFAP or mGFAP-Halo (R239C or R236H, respectively), and pictures were taken 24 h later. In the case of the mGFAP-Halo constructs, cells were incubated with the

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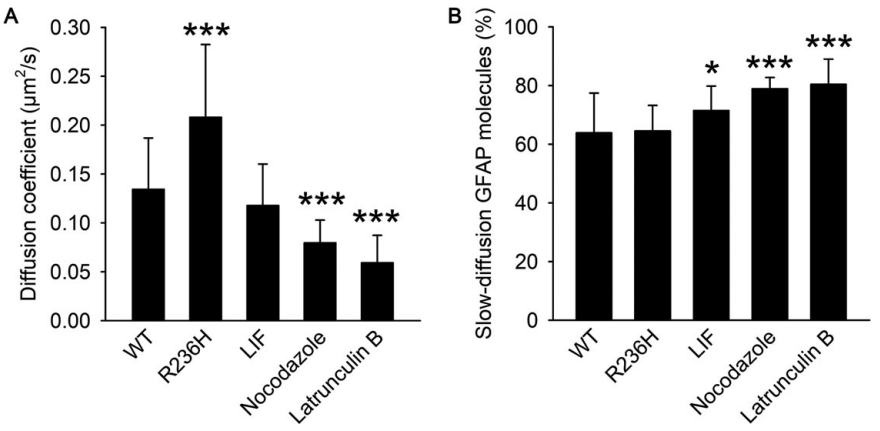

Figure 3. Genetic or pharmacological interference with the diffusion properties of mGFAP molecules. Average diffusion coefficient of rapid-diffusion molecules (A) and fraction of slow-diffusion GFAP molecules (B) calculated for individual cells (20 cells/group) by means of the Spot-On online tool (https://spoton.berkeley.edu/SPTGUI/docs/latest). The AxD-related mutation R236H or incubation of cells with Leukemia Inhibitory Factor (LIF, 100 ng/ml), Nocodazole (10 μM) or Latrunculin B (10 μM) for 2 hours changed the diffusion properties of single mGFAP molecules. LIF is a cytokine that induces the expression and polymerization of GFAP; Nocodazole is a drug that interferes with the formation of microtubules; and Latrunculin B is a drug that disrupts actin filaments. *, significant *versus* WT, $p < 0.05$; *** $p < 0.001$.

construct is more sensitive to the biological background and does not aggregate when mGFAP is mutated, but it allows a wider spectrum of applications, including super-resolution, single-molecule and protein–protein interaction analyses in living cells. We hope these new tools help the GFAP and astrocyte community to advance our understanding of physiological and pathological functions of this intermediate filament in living cells.

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Author contributions. RLV and RQ had equal contribution to this work. RLV developed the EGFP-hGFAP construct and carried out the experiments related to this construct (formation of aggregates and prevention by anti-amyloidogenic drugs). RQ developed the mGFAP-Halo construct and carried out the experiments related to this construct (single-molecule and SRRF analyses). Both of them worked in close collaboration at all cloning and mutagenesis steps. FM tested the formation of GFAP fibrils in different cellular contexts. Alvaro Crevenna and Zach Hensel supervised imaging experiments by RQ and the subsequent data analysis. FH supervised experiments by RLV and RQ, helped with statistical analysis and coordinated the team. FH and RQ wrote the main draft, with contributions from ZH and RLV.

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JF647 Halo ligand (100 nM) prior to imaging. WT hGFAP or mGFAP assembled into filaments (left panels), but AxD mutations changed this pattern (right panels). hGFAP R239C produced protein aggregates in the cytoplasm, while mGFAP R236H formed a homogeneous pattern throughout the cytoplasm without apparent filament structures. B-C, Quantification of the various patterns observed in U251 cells transfected with (B) WT EGFP-hGFAP (black bars) and mutant EGFP-hGFAP (white bars) or (C) the equivalent mGFAP-Halo constructs. **, significant *vs* WT, $p < 0.01$; ***, $p < 0.001$. D, Quantification of U251 cells transfected with the EGFP-hGFAP R239C mutant that displayed a normal filamentous network (black bars) or aggregates (white bars) after treatment with increasing concentrations (0.1–10 μM) of the neuroprotective compound CNB-001. All groups were statistically significant versus WT EGFP-hGFAP, $p < 0.001$. #, significant vs EGFP-hGFAP R239C, $p < 0.05$; ###, $p < 0.001$. E, Western blots showing similar levels of expression in U251 cells transfected with hGFAP-EGFP and mGFAP-Halo constructs.

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Data availability statement. The data that support the findings of this study will be made available in a public repository upon acceptance of the manuscript.

Conflicts of interest. The authors declare that they have no conflicts of interests.

Abbreviations:

hGFAP	human glial fibrillary acidic protein;
mGFAP	mouse glial fibrillary acidic protein;
EGFP	enhanced green fluorescent protein;
AxD	Alexander disease;
LIF	Leukemia Inhibitory Factor;
SRRF	Super-Resolution Radial Fluctuations.

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Peer Reviews

Reviewing editor: Dr. Michael Nevels

University of St Andrews, Biomolecular Sciences Building, Fife, United Kingdom of Great Britain and Northern Ireland, KY16 9ST

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and met required revisions.

doi:10.1017/exp.2020.1.pr1

Review 1: New tools for the visualization of glial fibrillary acidic protein in living cells

Reviewer: Albee Messing

University of Wisconsin Madison

Date of review: 9 December 2019
Published online:

Conflict of interest statement. Reviewer declares none.

Comments to the Author: Previous tagged versions of GFAP have mixed reputations, with some arguing that the tags interfere with normal function. These new versions may offer better options. But exactly where in the hGFAP sequence does the tag reside? Could the difference in aggregation between the two constructs derive from different levels of expression?

Score Card

Presentation

4.1 /5

Is the article written in clear and proper English? (30%)	4/5
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Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5

doi:10.1017/exp.2020.1.pr2

Review 2: New tools for the visualization of glial fibrillary acidic protein in living cells

Reviewer: Dr. Yu-Feng Wang

Date of review: 12 December 2019
Published online:

Conflict of interest statement. No

Comments to the Author: GFAP is not only a structural protein but also a functional molecule. However, evidence supporting a dynamic interaction between GFAP and other molecules remains to be collected in health and disease. The preparation of halo-tagged GFAP is clearly a powerful tool for exploring the mechanisms underlying GFAP plasticity under physiological and pathological conditions. Certainly, readers may expect to see full investigation and discussion of the molecular mechanisms for the differential expressions of the tagged GFAP under different conditions. That needs to consider not only potential spatial obstruction of the tags for the formation of GFAP dimers, tetramers and the filaments, but also for the cellular environments that are responsible for GFAP expression (see review by Li et al, 2019, Glia).

Score Card

Presentation

4.0 /5

Is the article written in clear and proper English? (30%)	4/5
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Context

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Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

4.4 /5

Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5