

# A NeuN specific fluorescent probe revealing neuronal nuclei protein and nuclear acids association in living neurons under STED nanoscopy

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Neuronal nuclei protein (NeuN) is a key RNA-associated protein to guide the transcription of RNA and regulate mRNA splicing in neurons. However, the lack of effective labelling and tracking method has hindered the elucidation of the biological mechanism of NeuN operation in living neurons to understand correlated central nervous system disorders. Here we report a molecular probe that can insert into a neighbouring hydrophobic-hydrophilic region in NeuN, which upon binding becomes capable of emitting light in the infrared. The NeuN specificity enables the probe imaging neuronal cells in primary brain regions including hippocampus, cerebellum, midbrain and cingulate gyrus. The probes optical properties are such to enable stimulated emission depletion (STED) imaging showing for the first time the 3D structure of RNA tangling into NeuN in a living neuron with tens of nanometer resolution.

## Introduction

Neurons are the main component of the central nervous system (CNS) and they are well organized into complex brain structures including hippocampus, cerebellum and cingulate gyrus, responsible triggering the basic brain functions such as cognitive and emotional through electrical or chemical signals.<sup>[1]</sup> hence, effective neuron labelling remains a powerful tool to further understand the relevant functions of neurons in different brain domains. Up to date, well-established methods such as Immunofluorescence,<sup>[2]</sup> lipophilic tracers such as (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) <sup>[3]</sup> and viral vectors<sup>[4]</sup> have been widely employed to label neurons for correlated investigation, however these approaches are often time consuming and pose risk to infect primary tissues with poor neuronal specificity. A recent effort was attempted using high-throughput screening to identify neuronal probes but the actual neuronal targeting mechanism remains unclear.<sup>[5]</sup>

We tackled this focusing our attention to a neuronal over-expressed protein, the neuronal nuclei protein (NeuN, also known as RBFOX3).<sup>[6]</sup> NeuN has been extensively used as mature neuron marker target (e.g. monoclonal antibodies A60) that specifically expressed only in neuronal nuclei and to some extent the cytoplasm of neuronal cells for most vertebrates,<sup>[7]</sup> and to measure the neuron/glia ratio in brain regions.<sup>[8]</sup> NeuN plays a prominent role in neural tissue development and regulation of adult brain function, while dysfunction or down regulation of NeuN have been associated with

several neurological disorders.<sup>[9]</sup> Although NeuN is a nucleic acid binding protein,<sup>[10]</sup> its association with DNA or RNA has never been visualized by imaging. Therefore, staining live neurons *in vivo* or *ex vivo* by employing a small molecular probe with high specificity under fluorescent microscopy as well as its extended SR microscopy, could be a valuable approach for researchers.<sup>[11]</sup>

In this work, we reported a labelling method with a rationally designed molecular probe **SL-Neu** that specifically binds to NeuN in neurons. The probe fluorescence is only activated upon binding where the protein inhibits its configuration transfer and enhances infrared emission and two-photon absorption. The high selectivity of **SL-Neu** towards neuronal tissue and NeuN protein in contrast to other parenchymal cells was demonstrated. Due to its superb photo-stability against depletion laser power, we also showed for the first time the nuclear acid association (RNA and DNA) with NeuN protein in living neurons under 3-dimensional stimulated emission depletion nanoscopy (3D-STED). Such functional probe not only offers a powerful tool for specifically isolation neuronal cells, but also provides an insight on visualization and investigation of precise NeuN activities and correlated CNS disease for neurobiology.

## Results and discussion

The chemical structures of the sulfosalt derivatives with D- $\pi$ -A (D = donor, A = acceptor) model including **SL-Neu**, **SL-1**, **SL-2** and **SL-3** were shown in Figure 1a. Their detailed synthesis, characterizations and crystal structural information were detailed in the ESI (Scheme S1, Figure S1-S15, Table S1-S3). Their photophysical properties, including nonlinear optical properties in the near-infrared region, were also systemically investigated (Figure S16-S22, Table S4-S5). It was found that the largest two-photon (2P) action cross-sections of probe **SL-Neu** in PBS buffer solution were located in the range of 840 – 940 nm. The dual-hydroxyl groups in **SL-Neu** molecule have a tendency to form strong hydrogen-bond (H-bond) with NeuN amino residues. Furthermore, the introduction of sulfo-group favors water solubility (Figure S14) and biocompatibility of the compounds, which in turn allowed avoiding the use of biological unfriendly

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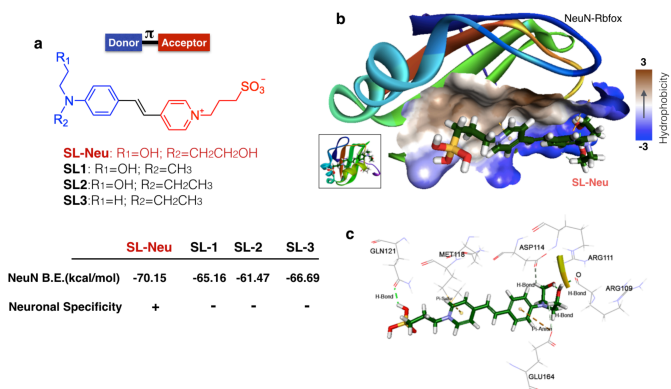
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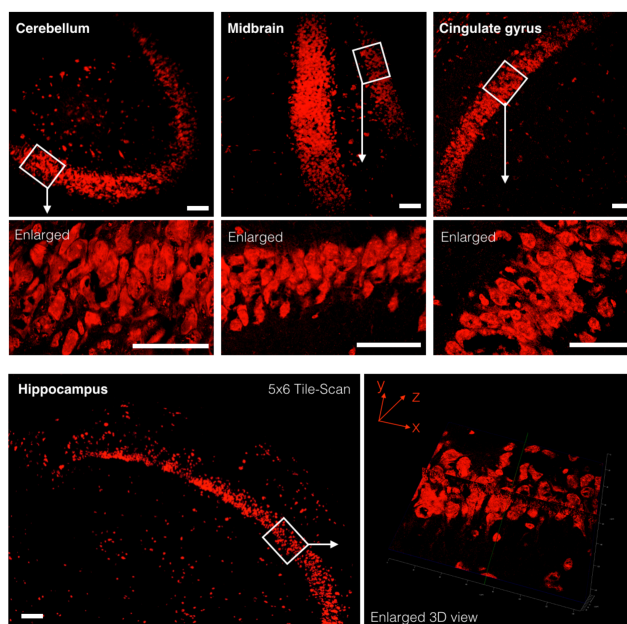
solvents (e.g. DMSO) and at the same time offering the possibility to form H-bond with NeuN protein, restricting molecular rotation.



**Fig. 1** (a) Chemical structure of sulfosalt derivatives including **SL-Neu**, **SL-1**, **SL-2** and **SL-3**; Insert table: modelled NeuN binding energy and live neuron specificity. (b) Molecular docking simulated hydrophobic and hydrophilic interaction of between **SL-Neu** and partial NeuN-Rbfox with indication of protein hydrophobicity; Insert figure: Molecular docking simulated diagram of **SL-Neu** and whole NeuN-Rbfox. (c) The detailed interaction of **SL-Neu** and amino acid residue in NeuN protein.

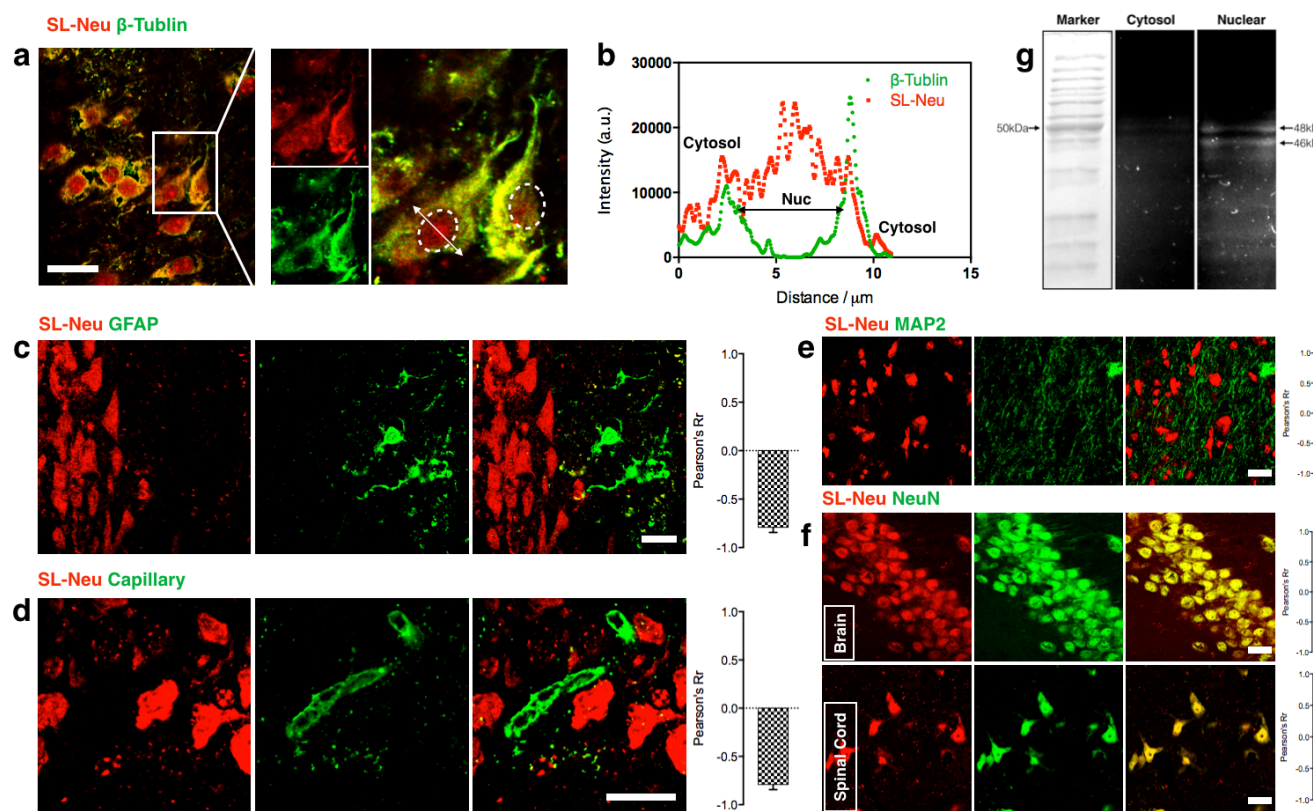
The specific recognition of **SL-Neu** and other compounds against NeuN-Rbfox protein (PDB: 2N82)<sup>13</sup> was initially confirmed *via* molecular docking using the Discover Studio ligandfitVina software (version 2016, The Biovia Co.).<sup>14</sup> The 2N82 PDB structure possess a semi-open cavity consist by a  $\alpha$ -helix and a  $\beta$ -sheet, offering the possible binding site for **SL-Neu** molecule (Figure 1b and Figure S23). In addition, the hydrophobic (Brown) hydrophilic (Blue) region in the protein active site (Figure 1b) interacts with corresponding hydrophobic/hydrophilic moieties of **SL-Neu**. Indeed, the detailed interactions including hydrogen binding and  $\pi$ -sulfur,  $\pi$ -anion (Figure 1c and Figure S24) was clearly presented (MET118, GLU164, GLN121, ASP114, ARG109). Notably, the relatively higher binding energy (-70.159 Kcal/mol) and neuronal specificity from **SL-Neu** molecule (Figure 1a insert table and Table S6) might be contributed by the dual-hydroxyl moieties, together with sulfonic acid group which led the formation of a 'triangle-like' H-bond force within NeuN-Rbfox protein. Subsequently, the restricted rotation of such D- $\pi$ -A molecule may also led to enhanced intermolecular charge

transfer (ICT), similarly as the influence of its solvent viscosity (Figure S25-S27). On the other hand, molecules **SL1**, **SL2** and **SL3**, which lack the dual-hydroxyl moieties, have no neuronal selectivity at later *ex vivo* experiments.



**Fig. 2** Confocal images of cerebellum, midbrain, cingulate gyrus and hippocampus regions of fresh mouse brain sections incubated with **SL-Neu** compound (10  $\mu$ M) for 30 min with high magnification micrographs of the white rectangular regions, with 3-dimension image from selected region from hippocampus. Scale bar = 100  $\mu$ m.

To elucidate the selectivity of these molecules for brain cells, fresh brain sections as well as liver, kidney and heart sections were prepared and incubated using **SL-Neu**, **SL-1**, **SL-2** and **SL-3** under 37  $^{\circ}$ C and 5 %  $\text{CO}_2$  for 1 hour. The primary tissue slices were then imaged under confocal laser scanning microscopy without further fixation. Notably, in liver, kidney and heart sections, the molecules produced negligible signal, while in **SL-Neu** treated brain sections some regions displayed strong emission (Figure S28). A similar brain uptake pattern was also observed under two-photon confocal microscopy (Figure S29). This result was also verified using prefixed tissues that were permeabilized with paraformaldehyde, suggesting that such specificity was present to both living and fixed samples.



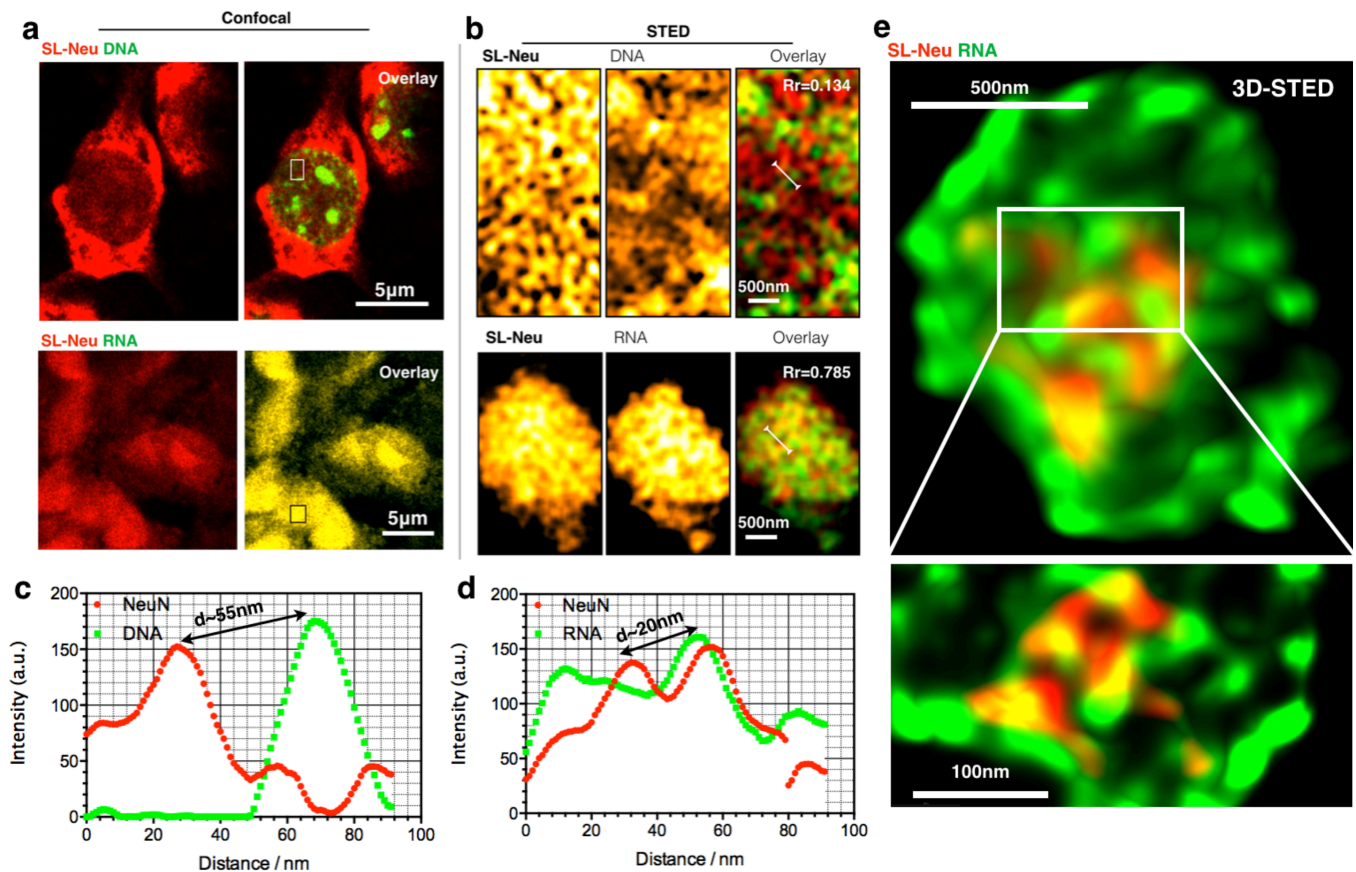
**Fig. 3** Colocations studies of SL-Neu compound. (a) Left: Colocations micrographs between  $\beta$ -Tublin and SL-Neu compounds. Right: The magnified micrographs from selected region. (b) Single cell SL-Neu and  $\beta$ -Tublin intensity profile indicated the cellular distribution (c-e) The SL-Neu-stained regions were immunostained with GFAP for astrocytes, AlexaFluor-488 Lectin for Capillary, MAP-2 for neuronal fibrils, respectively. (f) SL-Neu-stained regions of mouse brain and spinal cord were immunostained with NeuN antibody. (g) SDS-PAGE analysis of protein from mouse nerve cell extracted from fresh brain hippocampus upon staining with Coomassie brilliant blue R-250 and SL-Neu. Coomassie brilliant blue R-250 staining (left). SL-Neu molecules staining under ultraviolet transillumination. Scale bar = 20  $\mu$ m.

To further examine such specificity, brain sections with different neurons rich regions including cerebellum, midbrain, cingulate gyrus and hippocampus were evaluated. As Figure 2 and its enlarged micrographs showed, distinct signal was detected and these might highly correspondent to neuronal cells. Its neuronal specificity was confirmed when the brain cells were stained with the classic DNA dye DAPI (4', 6-diamidino-2-phenylindole), only cells within the hippocampus region displayed positive signal (Figure S30). In contrast, other cells (non-neuronal cells indicated by white arrows) showed negligible signals, suggesting the reduced uptake of SL-Neu molecules.

To verify the probe's intracellular distribution,  $\beta$ -Tublin antibody was used to label microtubules-rich neuron cells<sup>15</sup> after incubating with SL-Neu molecule. As displayed in Figure 3a in the magnified micrograph,  $\beta$ -Tublin demonstrated elongated neuronal fibril morphology and its signal were excluded from nuclear region. In contrast, SL-Neu displayed much less cytosolic distribution that

partially overlapped with  $\beta$ -Tublin, while it showed intense nuclear internalization. The uptake pattern was further confirmed as intensity plot profile as indicated in Figure 3b. Such cytosolic and nuclear observation is not surprising, since NeuN protein could be both expressed only in neuronal nuclei and to some extent the cytoplasm. This further supports that SL-Neu compound was NeuN specific.

In order to confirm the selectivity of SL-Neu against neuronal NeuN protein, a series of immunofluorescence experiments were performed to label brain parenchymal cells and another neuronal marker. As one of the most representative cell population in the brain, astrocytes can be effectively stained using glial fibrillary acidic protein (GFAP) antibody. Figure 3c demonstrated a few astrocytes closely associated with SL-Neu-positive cells from hippocampus region but without overlapping, as indicated by co-localization coefficient analysis *via* Pearson's Rr (right bar-chart). Brian



**Fig. 4** (a) Confocal images of live neuron treated with **SL-Neu** and NucRed (DNA), Syto-9(RNA). (b) The corresponding STED images showing the combination of **SL-Neu** and DNA, RNA respectively under high resolution. (c) and (d) The fluorescent intensity profile of the lines in chromatin region. (e) and (f) 3-dimension STED micrographs indicating the association of **SL-Neu** and RNA.

endothelial cell is the main component of blood brain barrier that exist within brain parenchyma, responsible for substances exchange between central nervous system and the rest of body. As Figure 3d shown **SL-Neu** positive cells display no co-localization with blood vessels, again suggesting a very high specificity of **SL-Neu** molecule towards a specific cell type, which might correspond to neurons. Subsequently, MAP2 (microtubule-associated protein 2, a common marker of neuronal axons) was used to specifically label neuronal fibrils, and showed little overlap with **SL-Neu** signal.

Based on the aforementioned results such as strong intracellular fluorescence of **SL-Neu** in neurons rich regions, high intensity in nuclear region of  $\beta$ -tubulin rich neuronal cells, non-specific interaction for non-neuronal cells and neuronal MAP2, co-staining with NeuN antibody was eventually performed. Here brain and spinal cord of mouse were harvested, their slices were prepared and incubated with **SL-Neu** followed with standard immunofluorescence protocol. Figure 3f clearly presents high degree of co-localization in both brain and spinal cord sections (Figure S31-S32). Generally, NeuN reactivity is mainly nuclear, whereas it may also be found in the cytoplasm of numerous neuronal cell types. Nonetheless, these immunofluorescence experiments strongly suggested **SL-Neu** molecules could effectively and specifically penetrate into live neuronal cells and internalize with neuronal nuclei protein, in a good agreement with our initial design purpose and computation modelling results.

To further confirm whether the staining of **SL-Neu** was indeed associated with the neuronal nuclei (NeuN) protein, nuclear and cytoplasmic proteins of mouse nerve cell were isolated from the hippocampus region, and protein electrophoresis was carried out. After electrophoresis, the gels were stained with **SL-Neu** (10  $\mu$ M) for 12 h, and Coomassie brilliant blue was used as a reference. As shown in Figure 3g, **SL-Neu** molecules manifested the staining specificity of NeuN protein distributed in nucleus and cytoplasm by detecting two major bands (nuclear) and two weak bands (cytoplasm), highly correspondent to NeuN/Rbfox46- and 48-kDa subtypes. This result was in good agreement with imaging result, which showed **SL-Neu** signal and the NeuN protein signal with a considerably high overlay rate, again strongly suggesting **SL-Neu** was a NeuN specific fluorescent probe in either live or fixed neuronal cells.

The utilization of **SL-Neu** molecules under stimulated emission depletion (STED) nanoscopy to reveal the association between NeuN protein and nuclear acid was also performed. Although some literature data suggests that NeuN is a pre-mRNA alternative splicing regulator and binds to RNA,<sup>10</sup> the correlation between NeuN and DNA or RNA in living neurons under super-resolution nanoscopy has never been demonstrated elsewhere, due to the lack of effective probes. Since STED technique require intense normal confocal laser along with a depletion laser, photo-stability is critical,<sup>16</sup> hence **SL-Neu** photo-resistance under STED condition was evaluated. Live brain section incubated with **SL-Neu** and exposed under STED laser over 400-times continued scans. Intensity profile



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suggested such compound displayed super-stability under both confocal and STED condition (Figure S33).

Subsequently, in order to display the ultra-detailed correlations between **SL-Neu** and nuclear acid, we performed co-localization experiments using commercial dyes NucRed and Syto9 for DNA and RNA, respectively. Conventional live cell imaging was firstly obtained, suggesting the compatibility of **SL-Neu** molecules with those nuclear acids marker under confocal microscopy (Figure 4a and Figure S34). Due to the remarkable photo-stability and red emission of **SL-Neu** molecules, STED super resolution micrographs were captured. Figure 4b indicated the nuclear NeuN protein was successfully marked with high signal-to-noise contrast. Neighboring chromatin structures were also revealed and the corresponding x-y plot profile indicating a ~55 nm distance between NeuN and DNA. In contrast, RNA showed much more overlapping signal with less than ~20 nm distance was verified. Indeed, 3D super resolution single nucleolar imaging, with 85 Z-sections (Figure 4e), was successfully obtained and indicated the actual binding details of NeuN protein within the RNA entangled nucleoli. In contrast, DNA displayed much less interaction with **SL-Neu** signal (Figure S35). Although previous studies suggested NeuN protein could internalize with DNA *in vitro*, our observation using **SL-Neu** as a probe might strengthen that NeuN protein is a RNA associate protein in living neurons.

## Conclusions

In summary, we have rationally designed a small fluorescent probe **SL-Neu** that can selectively target live neuron NeuN protein. **SL-Neu** live neuron labeling coupled with NeuN binding properties rendered further observation of NeuN and nuclear acid association using super-resolution technique under STED nanoscopy. This innovative compound serves as an imaging tool for living neurons as well as to shed light to correlated cellular mechanisms and ultimately NeuN associated CNS diseases.

## Conflicts of interest

There are no conflicts to declare.

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