Neuron Imaging

DOI: 10.1002/ange.201408614

NeuO: a Fluorescent Chemical Probe for Live Neuron Labeling**

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Abstract: *To address existing limitations in live neuron imaging, we have developed* ***NeuO****, a novel cell-permeable fluorescent probe with an unprecedented ability to label and image live neurons selectively over other cells in the brain.* ***NeuO*** *enables robust live neuron imaging and isolation in vivo and in vitro across species; its versatility and ease of use sets the basis for its development in a myriad of neuronal targeting applications.*

Neurons are responsible for information processing and transmission in the brain by electrical and chemical signals. They are organized into complex neural networks that underlie the basic brain functions (e.g., cognitive, emotional, and motor).[1] Over the past century, various tools have been developed to label neurons. However, these methods either lack intrinsic selectivity or compromise cell viability. Nissl and Golgi stains work only for frozen or fixed brain tissue preparations;[2] membrane staining dyes such as DiI and DiO are not selective for neurons and require manual injection for retrograde labeling.[3] On the other hand, neurotransmitter mimics work only for subtypes of neurons and under selective environments.[4] Expression of fluorescent proteins driven by neuron-specific promoters requires genetic manipulation which may induce undesirable effects to native protein functions and can be less straightforward.[5] Thus, a chemical probe that is widely applicable for generic staining of live neurons in vitro and in vivo would thus represent a valuable tool for researchers. Current methods to selectively isolate live neurons are based mainly on density centrifugation[6] or differential adhesion[7] protocols. The same selective probe can also be used with automated sorting methods for easy isolation of live neurons. Herein, we present **NeuO**, a live cell imaging probe that selectively stain neurons fluorescently in the presence of other brain cells. **NeuO** can be applied to multiple imaging and cellular platforms for the real-time imaging of neurons and is observed to be useful both in vitro and in vivo.

In view of the lack of mechanistic cues to rationally design probes for neurons, we envisioned that high-throughput screening will be necessary to assist us in identifying promising leads that fluorescently label live neurons.[8] A subset of 5040 fluorescent compounds was selected from our in-house diversity-oriented fluorescence libraries (DOFL; Supporting Information (SI), Figure S1). The dyes were then applied to a primary brain cell screening platform,[9] which employed mouse primary neurons, astrocytes, and microglia cultures isolated by differential adhesion methods from P1–P3 neonatal mouse brains (Figure S2).[7] These primary cells were prepared in 384-well microplates and cultured for 5 days in vitro before incubation with the DOFL compounds at 500 nM concentrations for 1 h. Through multiple rounds of automatic intensity-based analysis and image-based evaluation (Figure S3), we identified a lead fluorescent probe **1** that selectively stained neurons fluorescently (Table 1, Entry 1).

To improve the neuron selective response, a structure–activity relationships (SAR) study was initiated to evaluate the chemical groups important for conferring the dye's neuronal response. In total, 21 derivatives were prepared

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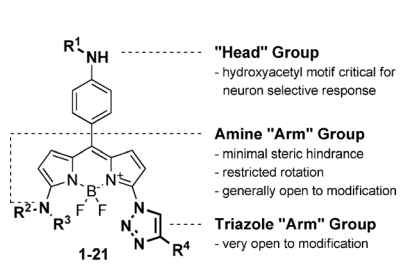
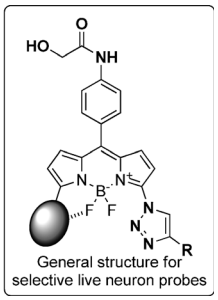
[**] This work was supported by an intramural funding from the A*STAR Biomedical Research Council and Singapore Ministry of Education Academic Research Fund Tier 2 (MOE2010-T2-1-025). J.C.E. was supported by a NGS Scholarship. C.L. was supported by an A*STAR Graduate Scholarship. Q.Y. was supported by a Duke-NUS SRP Phase 2 Research Block Grant given to H.S.J. D.S. thanks the G. Harold & Leila Y. Mathers Charitable Foundation. All animal work was carried out under the approved IACUCs no. 100564 and no. 100575 in compliance with NACLAR Guidelines.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201408614.

Table 1: Structure Activity Relationships.[a]





Dye	R^1	R^2	R^3	R^4	log *P*[b]	SLI
1[c]	(CO)CH_2OH	2-Me-C_6H_4-CH_2-	H	*s*Pe	4.5	17
2	(CO)CH_2OH	2-Me-C_6H_4-CH_2-	H	*n*Pr	3.2	23
3[d]	(CO)CH_2OH	Bn	H	*n*Pr	2.8	26
4	(CO)CH_2OH	Bn	H	*n*Hp	4.7	23
5	(CO)CH_2OH	Bn	H	*t*Bu	3.1	22
6	(CO)CH_2OH	Bn	H	Ph	3.3	20
7	(CO)CH_2OH	Bn	H	H	1.8	13
8	(CO)CH_2OH	2-MeO-C_6H_4-CH_2-	H	*n*Pr	3.0	19
9	(CO)CH_2OH	3-MeO-C_6H_4-CH_2-	H	*n*Pr	2.8	21
10	(CO)CH_2OH	4-MeO-C_6H_4-CH_2-	H	*n*Pr	2.7	19
11	(CO)CH_2OH	4-Me-C_6H_4-CH_2-	H	*n*Pr	3.2	17
12	(CO)CH_2OH	4-*t*Bu-C_6H_4-CH_2-	H	*n*Pr	4.2	11
13	(CO)CH_2OH	4-AcNH-C_6H_4-CH_2-	H	*n*Pr	1.4	24
14	(CO)CH_2OH	*n*Bu	H	*n*Pr	2.9	17
15	(CO)CH_2OH	H	H	*n*Pr	1.2	17
16	(CO)CH_2OH	Cy-CH_2-	H	*n*Pr	3.8	10
17	(CO)CH_2OH	Bn	Me	*n*Pr	3.1	8
18	Ac	Bn	H	*n*Pr	3.4	0
19	(CO)CH_2Cl	Bn	H	*n*Pr	3.8	0
20	(CO)CH_2OMe	Bn	H	*n*Pr	3.6	6
21	CH_2CH_2OH	Bn	H	*n*Pr	3.3	0

[a] Abbreviations: *s*Pe, *sec*-pentyl; *n*Hp, *n*-heptyl. [b] Relative hydrophobicity, log *P* as determined by the HPLC method (Table S2).[11] [c] Primary hit from DOFL screening. [d] **NeuO**.

which differ systematically in the chemical groups, namely, the acetyl "head", and the amino and triazolyl "arms" (Table 1). These fluorescent dyes were then assessed for their selectivity index (SLI), which is a normalized functional measure of the dye's selective staining of neurons over its nonselective staining (Table 1). Like the stain index,[10] SLI is defined as D/W, in which D is the difference between positive and negative populations and W is 2 standard deviations (S.D.) of the negative population (Figure 1 b, left); higher SLI compounds brightly stain live neurons more selectively and segregate neurons more distinctly on flow cytometry.

Our measurements revealed that compounds **2** to **7**—derivatives substituted with various alkyl and aryl moieties on the triazolyl "arm"—were able to sort neurons with excellent selectivities. Compounds **8**–**11** and **13**–**15**, which vary in the substituents on the "amino" arm, similarly showed high selectivities. However, compounds with bulky amino "arms", such as **12** and **16**, displayed largely reduced neuronal staining. Together, these observations suggest that both the triazolyl and amino "arms" can accommodate a range of chemical modifications with the former having greater scope than the latter.

On the other hand, compounds **18**–**21** and **23**, which carry various "head" groups, have significantly lower or no selectivity, highlighting the importance of the hydroxyacetyl for optimal neuron recognition. From the SAR, we may deduce that a compound having the general structure shown in Table 1 would make a feasible live neuron-selective probe. This information will be relevant to future probe designs for imaging live neurons. Compound **3**, which showed the highest selectivity toward live neurons, was thus chosen as the final fluorescent probe and named **Neu**ron **O**range (**NeuO**, λ_{ex}/λ_{em} = 470 nm/555 nm; Figure 1 a).

Within 1 h after application of 500 nM of **NeuO**, live neurons were seen to be selectively stained over other non-neuronal cells in the background (Figure 1 c, left), with a distinct cytoplasmic perinuclear staining pattern. In addition, high magnification imaging of **NeuO**-stained primary neuron cultures processes showed that **NeuO** staining extends to the fine processes of the neurites, with continuous dye staining throughout some parts of the neurite or otherwise being localized to discrete granules along the branches (Figure 1 c, right). Confocal imaging of **NeuO** demonstrates that the dye does not co-localize significantly with any of the known organellar trackers (Mitotracker, Lysotracker, ER tracker, Golgi tracker; Figure S5a). To confirm that the dye was selectively staining neurons over other neural cells, **NeuO**-stained cells (Figure 1 d) were fixed and immunostained with antibodies specific for neurons (β-III-tubulin), astrocytes (glial fibrillary acidic protein, GFAP), and oligodendrocytes (O4; Figure 1 e). This was further verified by analysis of characteristic neuronal gene expressions in the FACS-sorted populations of mixed primary neural cells from neonatal mouse brains separated using **NeuO** (Figure 1 b, middle); the isolated **NeuO**-positive cells expressed a high level of the neuronal marker, β-III-tubulin (Figure 1 b, right). Immunostaining with antibodies specific to other neuronal markers, including MAP2, NCAM, and NeuN, similarly supported neuronal identity (Figure S6b). **NeuO** also labeled most neuronal cell types including glutamatergic, GABAergic, cholinergic, dopaminergic, and serotinergic neurons as demonstrated by post-**NeuO** immunostaining with specific antibodies (Figure S5c). We tested whether **NeuO** labeling triggered any changes in cell viability or neuronal property. The application of 10 μM of **NeuO** to cultured neurons did not affect the cell survival rate when measured by the MTS assay (Figure S6a), nor neuronal morphology based on Scholl and Fractal analysis (Figure S6b).[12] Furthermore, **NeuO**-stained neurons showed indistinguishable changes in spiking pattern (Figure S7a and S7b), resting membrane potential (Figure S7c), capacitance (Figure S7d), and membrane resistance (Figure S7e), indicating that **NeuO** is not detrimental to neuron function.

NeuO exhibits excellent cell permeability due to its ability to diffuse rapidly into and stain neuronal cells passively upon loading into the cell media. In Video S1, mixed primary neural cell cultures isolated from P1–P3 neonatal brains were allowed to grow to confluence before staining with **NeuO** and **CDr10** (a microglia-selective dye)[9a] for dual color imaging. A large neuron was clearly visible amidst a dense bed of neuroglia and **NeuO** staining remained stable for up to 36 h.

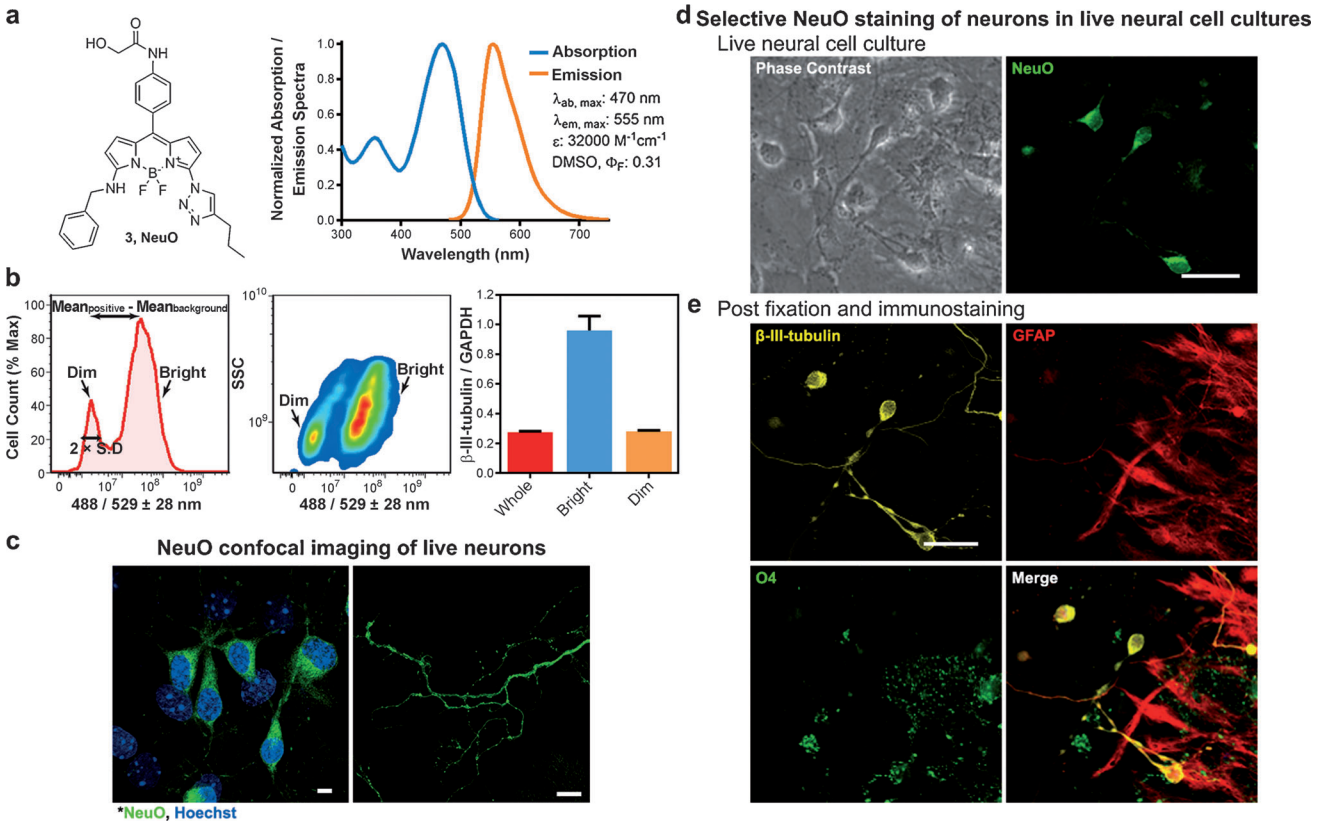


Figure 1. Properties of **NeuO**. a) Chemical structure and optical properties of **NeuO** (10 μM, DMSO). b) Left: neuronal population segregation by flow cytometry as represented by a histogram; calculation of SLI: $D/W = (mean_{positive} - mean_{background})/(2 \times S.D.)$. Middle: neuronal population segregation by flow cytometry as represented by a pseudocolor bivariate plot; *x* and *y*-axes are flow cytometry measures of the **NeuO** fluorescence signal (FL1, 530/30 nm) and granularity, respectively. Right: β-III-tubulin expression analyzed in the unsorted, bright, and dim fraction of cells **NeuO**-sorted neonatal mouse brain cells. c) Confocal images of live **NeuO**-stained neurons on a bed of astrocytes (left) and a high magnification image of a neuron dendrite (right); scale bar: 10 μm and 5 μm, respectively. d,e) Selective staining of neurons in live neural cell cultures using **NeuO**; d) phase contrast and fluorescence image of live mixed primary neural cell culture stained with 500 nM of **NeuO**; e) live **NeuO** stained cells were subsequently fixed and permeabilized for immunocytochemistry; **NeuO**-positive live cells were positive for the neuron marker β-III-tubulin, but not for other neural cell type markers (GFAP for astrocytes, O4 for oligodendrocytes); scale bar: 20 μm.

DIV3 primary rat hippocampal neurons cultured in the presence of **NeuO** also showed normal neurite formation in vitro (Video S2). In conditions requiring multiple washes, the dye is rapidly washed out from live neurons in the absence of excess dye (Figure S8a). However, neuronal staining remained intact after the stained cells were fixed by paraformaldehyde, although the dye staining was susceptible to cell permeabilization and organic solvent washing (Figure S8b). Hence **NeuO** staining is best applied in live neuron samples. This is in contrast with conventional Nissl and Golgi staining, which require membrane permeabilization by detergents before the stains can be visualized in neurons. However, **NeuO** is not selective for neurons in dead cells as seen from the dye staining all cells nonselectively post fixation (Figure S8b).

Two-photon ex vivo or in vivo imaging of the brain has become increasingly popular over the years due to its deep penetration with low photobleaching and phototoxicity. Two-photon imaging also provides high-resolution and efficient fluorescence detection,[13] thereby making it possible to image cells in the live brain tissue. To examine **NeuO** labeling in brain tissue, two-photon microscopy imaging was carried out in the hippocampal regions of mouse brain slices, because these regions are densely populated with glutamatergic neuronal cell bodies which can be easily identified by their characteristic s-shaped organization. **NeuO** densely labeled the principle cell layer of the DG, the *stratum granulosum*, and sparsely labeled the principle cell layer of CA1, the *stratum pyramidale* (Figure 2a). The cell labeling pattern was similar to P1 neonatal whole-brain staining through which clear neuronal structures were visible when viewed under confocal microscopy (Figure S9a). This staining pattern differed from the one of Sulforhodamine 101 (SFR 101), which stains live astrocytes (Figure S9a).[14] Neuron-selective staining was also effectively demonstrated in neurons derived from human embryonic stem cells, whereby **NeuO** positive cells were identified to be β-III tubulin-positive compared to **NeuO** negative cells which consisted mostly of S100β positive astrocytes (Figure S9b). In a 3 dpf zebrafish larvae, selective fluorescence staining of neurons by **NeuO** was observed in the neuromasts.[15] Neuromasts make up part of the lateral lines system in zebrafish, which are comprised of a set of rosette-

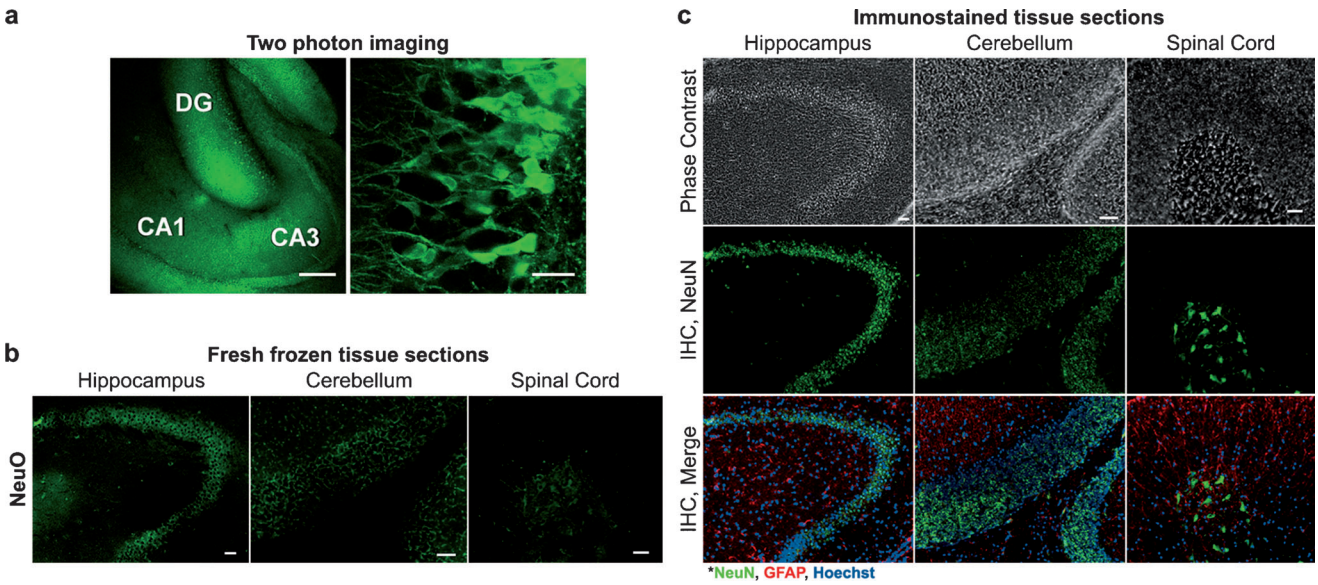


Figure 2. Applications of **NeuO**. a) Left: **NeuO** staining in the hippocampus shows dense labeling; scale bar: 200 μm. Right: **NeuO** staining of cell bodies in the dentate gyrus shows a perinuclear labeling pattern; scale bar: 20 μm. b) Images of the hippocampus, cerebellum, and spinal cord regions of the mouse brain showing neuronal staining after 1 h intravenous injection of **NeuO**; scale bar: 50 μm. c) The same **NeuO**-stained regions were immunostained with NeuN (green), GFAP (red), and Hoechst (blue). **NeuO**-stained regions correspond to NeuN immunostaining (green); scale bar: 50 μm.

like organs near the head and the body (Figure S9c).[16] These data demonstrate that **NeuO**'s neuronal staining is conserved across species.

To determine the selectivity of **NeuO** for neurons in vivo, the dye was administered by intravenous injection into the tail vein of mice. Brains and spinal cords were harvested at time points of 10 min, 1 h, 3 h, and 6 h and snap-frozen for microscopic analysis of frozen sections. Control images were also taken from non-injected mouse tissues at the same exposure (Figure S10a). The optimal time point was determined to be 1 h (Figure S10b) as observed from the improved neuron–glia signal ratio (Figure S10c). Brains isolated 1 h after **NeuO** injection showed clear fluorescence signals in fresh frozen tissue sections from the neurons of the hippocampus, cerebellum, and spinal cord (Figure 2b) with minimal background. Cell bodies of large neurons and their processes could also be observed in cross sections of the spinal cord. Immunostaining of the neuronal nuclear marker, NeuN, confirmed that the dye selectively stained neurons in the brain and spinal cord (Figure 2c). **NeuO** staining was also similar to the staining pattern of the Nissl dyes (Figure S10d). The successful detection of **NeuO** in the neurons of the brain and spinal cord demonstrates that the dye effectively bypasses the blood brain barrier and that neuronal staining is conserved when the dye is administered in vivo.

In conclusion, we described the systematic discovery of **NeuO** for the selective staining of live neurons of different species. **NeuO** represents the first selective fluorescent dye for specific live neuron labeling. The utility of **NeuO** for neuronal labeling has been demonstrated in a variety of imaging techniques and neuronal cell isolation procedures. Using **NeuO**, neurons can be selectively visualized and separated from mixed neural cell cultures. Overall, the discovery of **NeuO** provides a convenient tool for the selective labeling of live neurons which will be valuable for prospective studies in various areas of neuron development, networking, and degeneration.

Received: August 27, 2014
Revised: November 11, 2014
Published online: January 7, 2015

Keywords: BODIPY · fluorescent probes · imaging agents · live neurons

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