VIP Fluorescent Probes Very Important Paper

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Visualizing Microglia with a Fluorescence Turn-On Ugt1a7c Substrate

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Abstract: *Microglia, the brain-resident macrophage, are involved in brain development and contribute to the progression of neural disorders. Despite the importance of microglia, imaging of live microglia at a cellular resolution has been limited to transgenic mice. Efforts have therefore been dedicated to developing new methods for microglia detection and imaging. Using a thorough structure–activity relationships study, we developed CDr20, a high-performance fluorogenic chemical probe that enables the visualization of microglia both in vitro and in vivo. Using a genome-scale CRISPR-Cas9 knockout screen, the UDP-glucuronosyltransferase Ugt1a7c was identified as the target of CDr20. The glucuronidation of CDr20 by Ugt1a7c in microglia produces fluorescence.*

Microglia, the brain-resident macrophage, are known to be involved in brain development and to contribute to the progression of neural disorders, such as stroke, autism, and Alzheimer's disease.[1,2] Despite growing interest, microglia examinations have been restricted to either postmortem CNS tissue histopathology or fluorescent imaging in transgenic mice that express fluorescent proteins from microglia lineages.[3] Therefore, efforts have been dedicated to developing methods that can identify microglia populations.

Isolectin B4 (IB4), the plant lectin from *Griffonia simplicifolia*, has been widely used to identify microglia in both live and fixed brain tissues.[4] However, due to its strong affinity for perivascular cells and some neurons, IB4-based staining is not very specific for microglia.[5,6] The translocator protein (TSPO) has been also utilized as a pharmacological target to label activated microglia.[7] However, chemical ligands of TSPO are not ideal for labeling pan-microglial populations, as TSPO is ubiquitously expressed in the brain under normal conditions.[7]

Therefore, we previously developed CDr10 using diversity-oriented fluorescent molecule libraries (DOFLs).[8] However, CDr10 failed to label microglia in brain tissues due to the less stringent conditions of cell-based screening using purified microglia.[8] In this study, we applied a focused fluorescent library to confluent cultures of mouse primary glial populations containing microglia, astrocytes, and oligodendrocytes with neurons,[9] which provided a more stringent condition to identify suitable microglia probes that might work in vivo (Figure 1 A). We screened 880 chemical probes derived from monostyryl-containing asymmetric BODIPY backbones (BDs), from which CDr10 was derived.[10] Using unbiased library screening, we identified one derivative named CDr20 (cell designation red 20; Abs/Emi: 568/600 nm in MeOH; ε: 53 547 M^{-1} cm^{-1}) that clearly overlapped with *Csf1r*–EGFP signals; EGFP is expressed in microglia (Figure 1 B).[11]

Because CDr20 has a 3-hydroxy-4-methoxystyryl moiety, we evaluated the combinatorial structure–activity relationship profiles of 36 related styryl structures using epi-fluorescence imaging and flow cytometry analysis (Supporting Information, Table S1 and Figures S1 and S2). A hydroxyl group in any position of the benzene moiety (compounds **2**–**4**) was indispensable for labeling microglia by increasing the fluorescence

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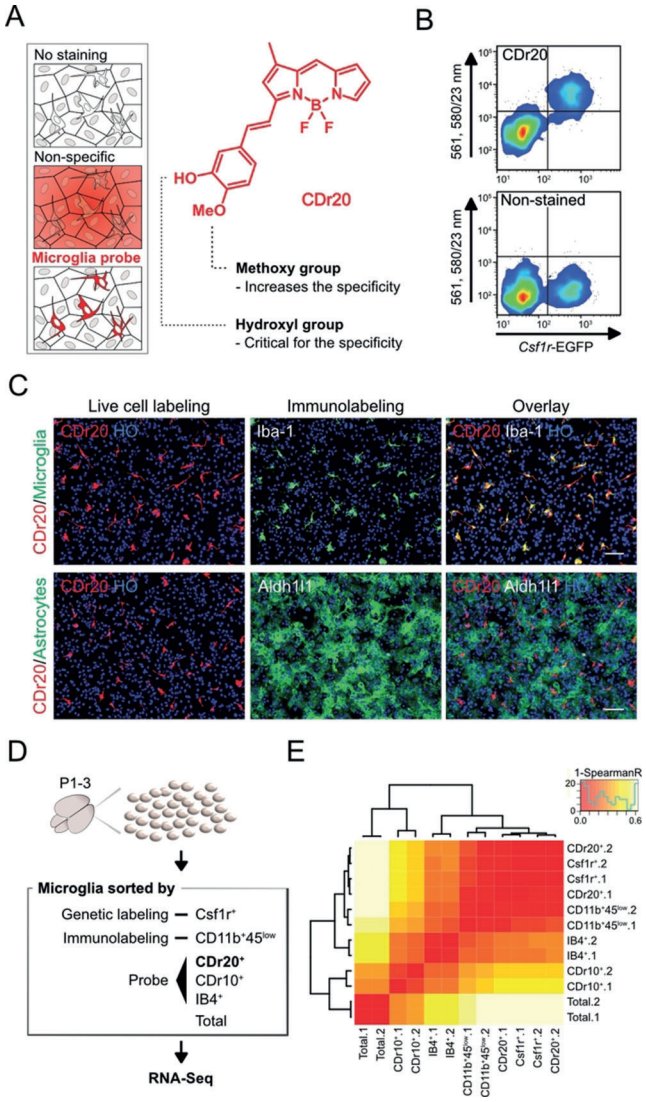

Figure 1. Identification of CDr20 as a microglia-specific fluorescent probe. A) Left: Experimental setup. Right: Chemical structure of CDr20 with the structure–activity relationships. B) Densitometry of non-stained or CDr20-stained *Csf1r*–EGFP brain cells. C) Superimposed images of CDr20 live-cell labeling and the indicated immunolabeling in primary cultured glial cells. Scale bars = 100 μm. D) Experimental scheme of the RNA-Seq analysis using the five different microglia sorting methods. Total, acutely isolated brain cells were used. E) Spearman's rank correlation coefficients of the gene expression values between the independent replicates of (D). Gating details are in Figure S16 in the Supporting Information. $CD11b^{+}/45^{low}$, CD11b-positive, and CD45-low. All experiments were using live cells, except for the immunolabeling shown in (C).

signal-to-noise (S/N) ratios. Other compounds without a hydroxyl group did not show any specificity to microglia. More hydroxyl groups may not be essential for labeling microglia. For instance, compounds **11** and **12** exhibited better specificity in labeling microglia, while compounds **13**–**15** and **29** did not. As demonstrated by compounds **16**–**20**, a methoxy group with an additional hydroxyl group improved the specificity for labeling microglia. Based on these findings, we selected CDr20 as the best microglia probe due to its stability (Figure 1 A). The specificity of CDr20 was further confirmed by the superposition of CDr20-stained live cells on Iba-1^{+}-microglia (97.3 %) and $GFAP^{+}$- (0.4 %) or $Aldh1l1^{+}$-astrocytes (0.0 % of live cells overlaid to the immunostained cells; Figure 1 C and Supporting Information, Figure S3). Interestingly, CDr20 did not emit a fluorescence signal in an aqueous environment, which enabled time-lapse imaging of microglia without removing CDr20 from the culture media (Supporting Information, Figure S4A and Video S1).

Due to its high S/N ratio in fluorescent imaging, we evaluated CDr20-based fluorescent-activated cell sorting (FACS). Isolated cell populations based on CDr20 fluorescence expressed high levels of microglia gene markers, such as *P2ry12*, *Csf1r*, and *Cx3cr1*, suggesting that single-probe staining is sufficient to isolate microglia from heterogeneous cell populations (Supporting Information, Figure S4B,C and Data S1). Using acutely isolated mouse brain cells, we also compared the microglia sorting efficiency of CDr20 to that of other conventional methods (Figure 1 D,E and Supporting Information, Table S2). We used *Csf1r*–EGFP-positive,[11] $CD11b^{+}/CD45^{low}$-gated,[12] and fluorescence-dye-conjugated-IB4-stained[4] cells as experimental controls, whereas CDr10-stained[8] and unsorted total live cells were used as the comparison groups (Figure 1 D). Gene expression analyses revealed that IB4- and CDr10-stained sorted cells and total live cells were more heterogeneous because IB4 and CDr10 exhibited affinity for endothelial cells and other glial cells (Figure 1 E and Supporting Information, Figure S5A,C). In contrast, cells sorted on the basis of CDr20 fluorescent signal exhibited similar transcriptome profiles as those of microglia from *Csf1r*–EGFP mice and $CD11b^{+}/CD45^{low}$-gated cells (Figure 1 E and Supporting Information, Figure S5B,C), indicating that CDr20-based FACS can be used to isolate microglia populations.

Next, we tested whether CDr20 could label microglia populations in brain tissues using live-cell, confocal imaging. Compared to IB4-based microglia staining, CDr20 did not stain the blood vessels in the brain slices (Figure 2 A and Supporting Information, Figure S6), and its intracellular fluorescence signal was clear in identifying microglia (Figure 2 B). A total of 94.1 % of *Csf1r*–$EGFP^{+}$ mouse microglia in the brain slices of adult mice were labeled by CDr20 (Figure 2 B and Supporting Information, Figure S7A). To test its ability to label microglia in an intact brain, a whole, live *Csf1r*–EGFP day 13 mouse embryo was incubated with CDr20 (Figure 2 C). CDr20 noninvasively labeled microglia in the live embryo (Figures 2 C); 99.2 % of $EGFP^{+}$ microglia were labeled by CDr20 (Supporting Information, Figure S7B). To further test whether intravenous administration of CDr20 could label microglia in vivo, we performed a craniotomy above the primary somatosensory cortex in mice (Supporting Information, Figure S8). Intravenous infusion of CDr20 resulted in CDr20 fluorescence signals on the EGFP-expressing microglia of neurological models, mannitol-injected wild-type (Figure 2 E and Supporting Information, Figure S9), and on the microglia of aged Alzheimer's disease mice (Supporting Information, Video S2), suggesting that CDr20 can label microglia in vivo.

To identify proteins that confer the functionality of CDr20 in microglia, we performed a genome-scale CRISPR-Cas9

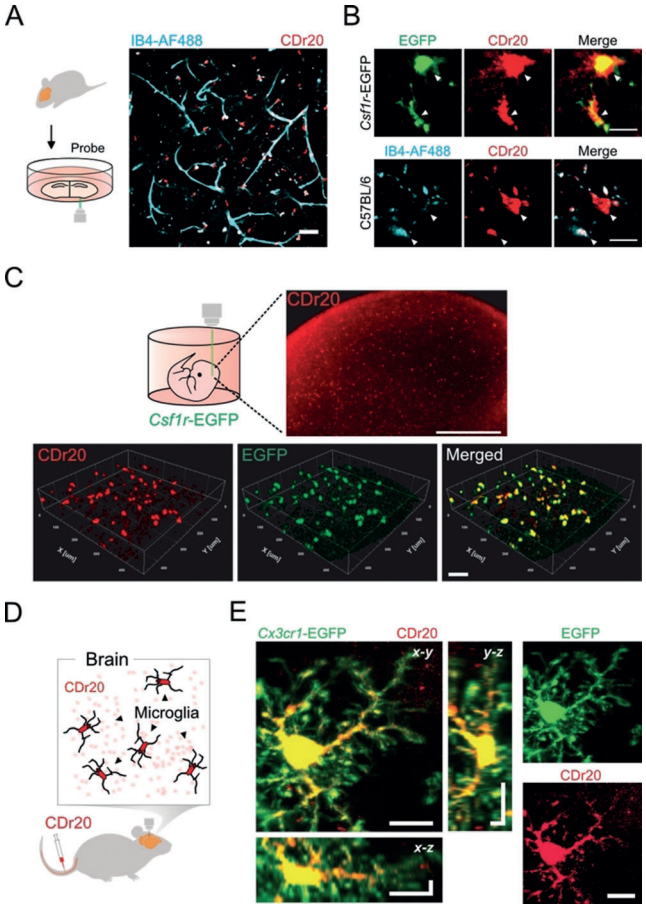

Figure 2. Two-photon ex vivo and in vivo microscopy of live microglia using CDr20. A) Left: Experimental setup. Right: Difference between Isolectin B4 (IB4)–Alexa Fluor 488 (AF488) and CDr20 in labeling blood vessels and microglia in an adult mouse brain slice (live). B) CDr20-labeled microglia colocalized with Csf*1r*–EGFP$^+$ (Top) or IB4–AF488$^+$ (Bottom) microglia (Arrowheads). C) Labeling of embryonic microglia by simple incubation of a whole live mouse embryo (day 13) with CDr20. Top: A low-magnification epi-fluorescence image. Bottom: Colocalization of CDr20-labeled cells with EGFP$^+$ microglia. D) Scheme of in vivo labeling. E) In vivo microglia imaging with CDr20 intravenous injections after systemic inflammation induced by intraperitoneal lipopolysaccharide injection. Left: 3D view, Right: Single-channel images. Scale bars = 50 µm in (A) and (C-bottom), 500 µm in (C-top), and 10 µm in (B) and (E).

knockout screening by transducing SpCas9-expressing BV2 microglial cells with the Brie library (Figure 3A).[13] The resulting single gene-knockout populations were subsequently labeled with CDr20, and cells with different CDr20-derived fluorescence intensities were further enriched by multiple rounds of FACS to separate CDr20-negative (CDr20$^-$) from CDr20-positive (CDr20$^+$) cells (Figure 3A). The appearance of CDr20$^-$ cell populations indicates that a gene is responsible for generating the fluorescence signal for CDr20 in microglia (Figure 3B and Supporting Information, Figure S10). The abundant single guide RNAs (sgRNAs) from the enriched CDr20$^-$ populations were then identified by comparing the counts of each sgRNA with those from the unselected library.[13] Three sgRNAs (88071539, 88095234, and 21658821) targeting the *Ugt1a9*/*Ugt1a7c*, *Ugt1a7c*, and *Olfr1361* genes, respectively, were repeatedly accumulated in

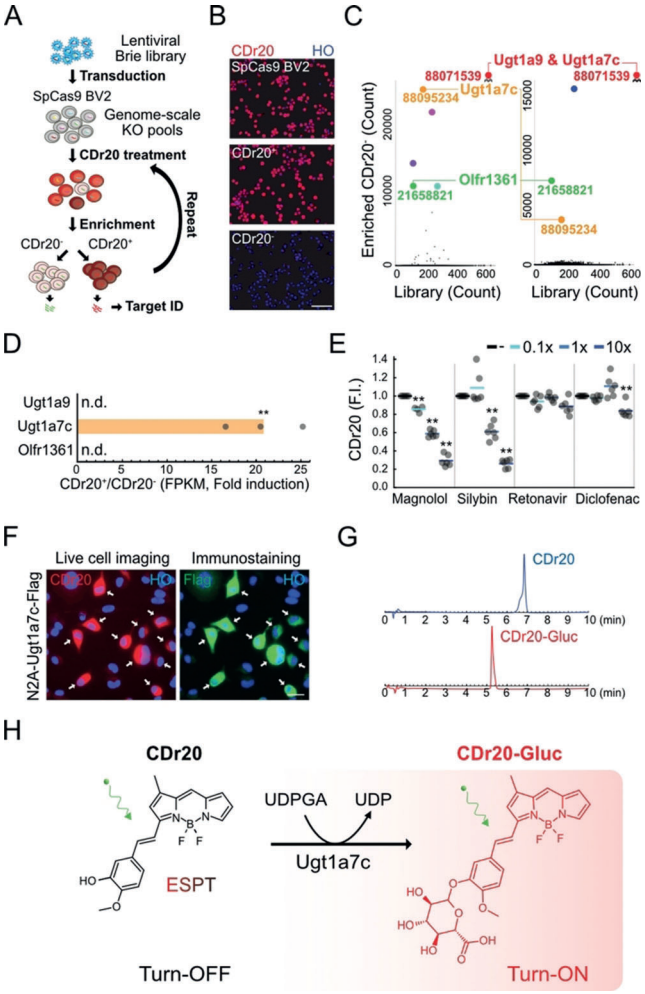

Figure 3. Identification of the turn-on fluorescence mechanism of CDr20 by Ugt1a7c. A) Schematic steps for the identification of CDr20 target genes with genome-scale knockout (KO) microglia. B) Images of CDr20-stained control and six-times enriched CDr20$^+$ and CDr20$^-$ populations. C) Dot plots of the read counts of the 78,637 sgRNAs in the library (*x*-axes) and CDr20$^-$ populations (*y*-axes). Number: library ID; 88071539 is enriched in outliers (left, 14000 k; right, 13200 k). Repeatedly observed sgRNAs are indicated with their target(s). D) The relative expression levels of the three candidate genes in CDr20$^+$ populations from other glia. n.d.: Not detected. E) Inhibition of microglial CDr20 staining by treatment of microglia with four Ugt inhibitors at 0.1, 1, and 10 times their IC_{50} concentrations. Bold lines: mean intensities. Dots: each data points (N = 4–6). F) Generation of the CDr20$^+$ population by Ugt1a7c–FLAG overexpression in Neuro2a (N2A) cells. Epi-fluorescence images. Arrows: Ugt1a7c–FLAG-expressing and CDr20-stained cells. G) LC-MS spectra of CDr20 (*m*/*z* 353.0) and CDr20–Gluc converted by a Ugt1a7c-containing microsome (*m*/*z* 531.1). H) The proposed fluorescence turn-on process of CDr20. ESPT: excited-state proton transfer. Scale bars = 100 µm (B) and 20 µm (F); **: $p < 0.01$.

CDr20$^-$ populations in two independent experiments (Figure 3C). Only *Ugt1a7c* had substantial expression levels (greater than 1 fragment per kilobase of exon model per million reads mapped, FPKM) and exhibited enhanced expression in CDr20$^+$ microglia among the candidate genes (Figure 3D). Interestingly, in the Ugt gene family, *Ugt1a7c* was the only member enriched in microglia (Supporting Information, Table S3). Previous transcriptomic analyses

have confirmed that the *Ugt1a7c* gene was expressed in a microglial-specific manner (accession number: GSE43808 and GSE52564) in the brain.[14,15]

Ugt1a7c protein is a UDP-glucuronosyltransferase with phenol-induced glucuronidase activity.[16] Since CDr20 contains a phenolic moiety essential for microglia labeling (Figure 1A), we tested whether the fluorogenicity of CDr20 in microglia was caused by the enzymatic activity of Ugt1a7c. Microglia displayed decreased CDr20-derived fluorescence upon either magnolol or silybin treatment, selective inhibitors of the Ugt1a7c human isoform,[16,17] but not upon ritonavir or diclofenac treatment, inhibitors of other Ugt1 family members (Figure 3E).[16,18] More importantly, ectopic overexpression of Ugt1a7c in a nonmicroglial cell line, Neuro2a (N2A), was sufficient to elicit CDr20 fluorescence (Figure 3F). Using LC-MS/HRMS, we observed that the glucuronidated form of CDr20 (CDr20–Gluc) in both CDr20 solutions, which had been treated with microsomal Ugt1a7c (Figure 3G and Supporting Information, Figure S11), and cell lysates from CDr20-treated Ugt1a7c-overexpressing N2A cells (Supporting Information, Figure S12).

Lastly, we examined how the conversion from CDr20 to CDr20–Gluc generated strong fluorescence signals in microglia. Quantum chemical calculations showed that CDr20 emission can be quenched through excited-state proton transfer (ESPT)[19] by losing one proton at the hydroxyl group upon photoexcitation (Supporting Information, Figure S13). However, ESPT can be effectively suppressed after CDr20 is converted to CDr20–Gluc (Figure 3H). Consequently, the fluorescence signal was enhanced due to in vitro conversion of CDr20 (quantum yield, $\Phi = 0.007$) into CDr20–Gluc ($\Phi = 0.611$) by Ugt1a7c (Supporting Information, Figure S14). In summary, we have discovered CDr20, a fluorogenic probe that labels microglia in both cell cultures and live rodent brains through its Ugt1a7c-mediated fluorescence turn-on process. CDr20 could be a useful tool for identifying microglia in neural disorders.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biological activity · fluorescent probes · imaging agents · microglia · structure–activity relationships

[1] M. Prinz, J. Priller, *Nat. Rev. Neurosci.* **2014**, *15*, 300 – 312.
[2] M. W. Salter, B. Stevens, *Nat. Med.* **2017**, *23*, 1018 – 1027.
[3] A. Chow, B. D. Brown, M. Merad, *Nat. Rev. Immunol.* **2011**, *11*, 788 – 798.
[4] W. J. Streit, G. W. Kreutzberg, *J. Neurocytol.* **1987**, *16*, 249 – 260.
[5] F. Boscia, C. L. Esposito, A. Casamassa, V. De Franciscis, L. Annunziato, L. Cerchia, *J. Neurochem.* **2013**, *126*, 428 – 436.
[6] T. Wälchli, J. M. Mateos, O. Weinman, D. Babic, L. Regli, S. P. Hoerstrup, H. Gerhardt, M. E. Schwab, J. Vogel, *Nat. Protoc.* **2015**, *10*, 53 – 74.
[7] G. J. Liu, R. J. Middleton, C. R. Hatty, W. W. Y. Kam, R. Chan, T. Pham, M. Harrison-Brown, E. Dodson, K. Veale, R. B. Banati, *Brain Pathol.* **2014**, *24*, 631 – 653.
[8] C. Leong, S. C. Lee, J. Ock, X. Li, P. See, S. J. Park, F. Ginhoux, S.-W. Yun, Y.-T. Chang, *Chem. Commun.* **2014**, *50*, 1089 – 1091.
[9] J. Saura, *J. Neuroinflammation* **2007**, *4*, 26.
[10] J. S. Lee, N. Y. Kang, K. K. Yun, A. Samanta, S. Feng, K. K. Hyeong, M. Vendrell, H. P. Jung, Y. T. Chang, *J. Am. Chem. Soc.* **2009**, *131*, 10077 – 10082.
[11] P. Wieghofer, K. P. Knobeloch, M. Prinz, *Glia* **2015**, *63*, 1 – 22.
[12] J. D. Sedgwick, S. Schwender, H. Imrich, R. Dörries, G. W. Butcher, V. ter Meulen, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7438 – 7442.
[13] J. G. Doench, N. Fusi, M. Sullender, M. Hegde, E. W. Vaimberg, K. F. Donovan, I. Smith, Z. Tothova, C. Wilen, R. Orchard, H. W. Virgin, J. Listgarten, D. E. Root, *Nat. Biotechnol.* **2016**, *34*, 184 – 191.
[14] C. Beutner, B. Linnartz-Gerlach, S. V. Schmidt, M. Beyer, M. R. Mallmann, A. Staratschek-Jox, J. L. Schultze, H. Neumann, *Glia* **2013**, *61*, 1429 – 1442.
[15] Y. Zhang, K. Chen, S. A. Sloan, M. L. Bennett, A. R. Scholze, S. O'Keeffe, H. P. Phatnani, P. Guarnieri, C. Caneda, N. Ruderisch, S. Deng, S. A. Liddelow, C. Zhang, R. Daneman, T. Maniatis, B. A. Barres, J. Q. Wu, *J. Neurosci.* **2014**, *34*, 11929 – 11947.
[16] B. T. Gufford, G. Chen, P. Lazarus, T. N. Graf, N. H. Oberlies, M. F. Paine, *Drug Metab. Dispos.* **2014**, *42*, 1675 – 1683.
[17] L. Zhu, G. Ge, H. Zhang, H. Liu, G. He, S. Liang, Y. Zhang, Z. Fang, P. Dong, M. Finel, L. Yang, *Drug Metab. Dispos.* **2012**, *40*, 529 – 538.
[18] S. Algeelani, N. Alam, M. A. Hossain, G. Mikus, D. J. Greenblatt, *Xenobiotica* **2017**, *47*, 1 – 6.
[19] L. M. Tolbert, K. M. Solntsev, *Acc. Chem. Res.* **2002**, *35*, 19 – 27.

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Communications

VIP

Fluorescent Probes

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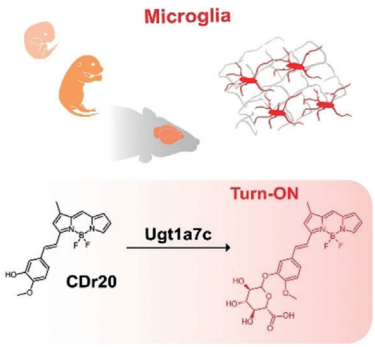
Visualizing Microglia with a Fluorescence Turn-On Ugt1a7c Substrate



Lightbulb moment: The fluorogenic probe, CDr20, specifically labels mouse microglia both in vitro and in vivo, enabling their visualization through a fluorescence turn-on response, mediated by the UDP-glucuronosyltransferase, Ugt1a7c. CDr20 could be useful for the characterization of the role of microglia during brain development and the pathogenesis of neural disorders.