Sudan Black B treatment uncovers the distribution of angiotensin-converting enzyme2 in nociceptors

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Abstract
Nervous system manifestations caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are of great concern. Neurological symptoms and the neurological effects induced by SARS-CoV-2, such as the loss of various sensory perceptions, indicate direct viral invasion into sensory neurons. Therefore, it is very important to identify the distribution of angiotensin-converting enzyme 2 (ACE2), the receptor of SARS-CoV-2, in human nervous system. However, autofluorescence from lipofuscin obviously impacted immuno-autofluorescence analysis in previous studies. We demonstrated that Sudan Black B (SBB) remarkably reduced the massive lipofuscin-like autofluorescence and the immuno-autofluorescence signal would be sharpened following the exposure compensation. Additionally, we confirmed that ACE2 was expressed in IB4+, CGRP+, and NF200+ sensory subpopulations. The mapping of ACE2 distribution in hDRG would facilitate the understanding of sensory disorder induced by SARS-CoV-2.

Keywords
COVID-19, ACE2, lipofuscin-like autofluorescence, SBB

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COVID-19 caused by SARS-CoV-2 has raged around the world, which is characterized by pneumonia, respiratory distress, and hypercoagulation. Many extrapulmonary manifestations of COVID-19 have been observed, of which neurological symptoms have driven increasing concern.1–3 These neurological symptoms are diverse, ranging from the loss of various sensory perceptions to the life-threatening acute disseminated encephalomyelitis.2 The detailed mechanisms of neurological symptoms in COVID-19 are still not explicit. However, direct viral invasion has been considered one of the key pathogenic pathways, and sensory-specific neurological effects including joint pain, headache, loss of smell, taste and chemesthesis, etc. suggest the involvement of sensory neural system.1 Many pre-clinical studies have shown direct coronavirus invasion in the nervous tissues and explored the distribution of angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, in the nervous system.4–6 Meanwhile, relative enrichment of ACE2 expression was detected in nasal epithelial cells from human lung tissue.7,8 This was consistent with the common hypogeusia and hyposmia symptoms of COVID-19. Therefore, it is very important to further identify the distribution of ACE2 in the human nervous system and many researchers have contributed to this. For example, Shiers et al.9 have detected ACE2 in human dorsal root ganglion (hDRG) and a subset of MRGPRD+ nociceptors co-expressed ACE2.9 However, we noticed that autofluorescence in immunofluorescent staining of human neural tissues was a universal

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problem disturbing the veracity of conclusions. The auto-
fluorescence should not be ignored while studying the dis-
tribution of ACE2, and a reliable method should be selected
to reduce its impacts.

Immunofluorescence analysis is distinctly affected by
biological autofluorescence, which generates in animal
tissues with age. Lipofuscin is a major source of auto-
fluorescence in cytoplasm of the nervous system, espe-
cially in human CNS. It is effective to some extent to
distinguish non-specific background fluorescence from
target fluorescence by setting up negative controls.
However, the presence of autofluorescence might still
disturb the statistical process. It had been illustrated that
preprocessing with 1–10 mM CuSO4 in 50 mM ammonium
acetate buffer (pH, 5.0) or Sudan Black B (SBB) in 70%
etanol is a practicable method to reduce lipofuscin-like
autofluorescence in 1999 by Schnell et al.10 SBB was also
proved to be more efficient to eliminate lipofuscin-like
autofluorescence in aged human CNS tissue. Following
experiments on the human brain, Oliveira et al.11 veri-
fi ed that SBB reduced auto-
fluorescence, however with an observable loss of intensity
of immunocytochemical target labeling. It is worth no-
icing that increasing exposure time when imaging helps in
recognizing fluorescent markers to some degree. For these
reasons, we believe applying SBB is worth trying to cut
down the influence of lipofuscin-like autofluorescence.

An experiment was designed to verify the e ffects of SBB,
and one hDRG was used (one male donor from the National
Human Brain Bank for Development and Function, Chinese
Academy of Medical Sciences, and Peking Union Medical
College (PUMC) in Beijing, China) to further determine the
distribution of ACE2 in sensory neurons. To certify if SBB
was appropriate for reducing autofluorescence and the
applicability of exposure compensation (EC) for the recog-
nition of fluorescence markers, three experiments were
performed. First, two sets of sections were performed with
the ordinary procedure of immunofluorescent staining, but
without any antibody incubation. One set was treated with
SBB (0.1% dissolved in 70% ethanol) for 5 min10 before
being coverslipped with VECTASHIELD Mounting Me-
dium with DAPI. Both sets were scanned at 488 nm and
594 nm stimulation, and exposure time was kept equal for all
sections treated with or without SBB. Second, sections were
incubated with primary antibody (rabbit anti-ACE2, Abcam,
ab15348) and secondary antibody (goat anti-rabbit IgG
Alexa Fluor® 488, Abcam, ab150077; Goat anti-Rabbit IgG
Alexa Fluor® 594, Abcam, ab150080) with SBB treatment
before being sealed. EC was applied to one set of sections
as scanned at 488 nm and 594 nm stimulation. Third, sections
were incubated with rabbit anti-ACE2 antibody or control
rabbit IgG for diaminobenzidine-coupled immunohisto-
chemistry to fundamentally eliminate the interference of
autofluorescence. Furthermore, hDRG sections were re-
spectively incubated with ACE2 antibody (rabbit anti-
ACE2, Abcam, ab15348) together with CGRP (calcitonin
gene-related peptide, goat anti-CGRP, Abcam, ab36001) or
NF200 (neurofilament 200, Chicken anti-NF200, Abcam,
ab4680). Then, sections were incubated with following
responding secondary antibodies or Isoclectin GS-IB4
Alexa Fluor 594 (Invitrogen, I21413) for 1 h: Donkey
anti-rabbit IgG Alexa Fluor 488 (Abcam, ab150073); donkey anti-goat IgG Alexa Fluor 594, Jackson
(Immu
oResearch, 705-585-147); and goat anti-chicken
IgY Alexa Fluor 594 (Abcam, ab150176).

Fluorescence was detected at both 488 nm and 594 nm,
which could be mostly erased by the application of SBB
under equal exposure time (Figures 1(a)–(d)). After in-
cubation with antibodies, strong signal was observed at
both 488 nm and 594 nm stimulation (Figure 1(e)). Only
weak but distinguishable signals were detected after treated
by SBB (Figure 1(f)). This phenomenon was consistent with previous reports and compensation of exposure time
helped capture sharpened images (Figure 1(g)). The per-
centage of positive cells was significantly decreased after
SBB treatment suggested that SBB prominently reduced
the positive signals induced by lipofuscin (Figures 1(h) and
(i)). In diaminobenzidine-coupled immunohistochemistry,
compared with the negative IgG control, clear ACE2
signals were detected after the application of rabbit anti-
ACE2 antibody suggesting the expression of ACE2 in
human sensory neurons (Figures 1(j) and (k)). In addition,
ACE2 was detected in IB4+ (marker for non-peptidergic
nociceptor), CGRP+ (marker for peptidergic nociceptor),
and NF200+ (marker for myelinated Aβ-fiber sensors)
sensory neurons (Figures 1(l)–(q)), suggesting the poten-
tial association between sensory ACE2 and COVID-
19-related pain.

SBB significantly reduced the massive lipofuscin-like
autofluorescence in negative control sections and ACE2
immunofluorescence staining sections. Exposure compen-
sation could offset the ACE2 signal weakened by SBB
treatment and would extend the application of SBB. Flu-
orescence staining and immunohistochemistry showed similar
distribution of ACE2 in hDRG. These above results pre-
liminarily verified that SBB reduced autofluorescence in
hDRG tissues while causing less impact on staining of target
molecule. Lipofuscin deposition occurs not only in the
nervous system but also in many other tissues.12 We believe
that attention should be paid to the fluorescence staining
experiments of old animal tissues, especially human CNS
tissues. Previous studies and our findings suggested that SBB
could be an applicable method for controlling auto-
fluorescence in CNS tissues.13 With the application of SBB,
we preliminarily confirmed the distribution of ACE2 in
hDRG and demonstrated the co-expression of ACE2 with
IB4+, CGRP+, and NF200+ sensory markers. We believe this
report contains important implications for further studies of
ACE2 distribution and understanding the legacy effects of
COVID-19.
Figure 1. Effect of Sudan Black B treatment on lipofuscin-like autofluorescence of hDRG. (A) Representative images of hDRG section stimulated by 488 nm and 594 nm without any antibody incubation. Scale bar: 100 μm. (B) Representative images of hDRG section stimulated by 488 nm and 594 nm without any antibody incubation and treated by Sudan Black B. Scale bar: 100 μm. (C and D) Treatment of Sudan Black B reduced the lipofuscin-like autofluorescence in hDRG section stimulated by 488 nm and 594 nm without any antibody incubation. ***p < 0.001, chi-square test, SBB versus Control. (E) Representative images of hDRG section stimulated by 488 nm and 594 nm incubated by rabbit anti-ACE2 antibody. Scale bar: 100 μm. (F) Representative images of hDRG section captured under equal exposure time incubated by rabbit anti-ACE2 antibody and treated by Sudan Black B. Scale bar: 100 μm. (G) Representative images of hDRG section captured with exposure compensation (EC) incubated by rabbit anti-ACE2 antibody and treated by Sudan Black B. Scale bar: 100 μm. (H–I) Treatment of Sudan Black B reduced the lipofuscin-like autofluorescence as immunofluorescence applied. ***p < 0.001, chi-square test, SBB+EC versus Control. ns, no significance, chi-square test, SBB versus SBB+EC. (J) Immunohistochemical staining showed the existence of ACE2 in hDRG. Scale bar: 100 μm. (K) Summation of ACE2+ cells detected by immunohistochemical staining in hDRG. (L–N) Representative microscopic images of immunostaining for ACE2 co-expression with CGRP, IB4, and NF200 in hDRG treated with SBB. Positive cells shown by arrows. Scale bar: 100 μm. (O–Q) Percentages of ACE2+ cells in hDRG with sensory neuronal markers (CGRP, IB4, and NF200). Scale bar: 100 μm.
Author Contributions
SS and NY designed and performed the experiments and then acquired the data. SS and HZ contributed to data analysis and interpretation. All authors read and approved the final manuscript.

Declaration of conflicting interests
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Ethical approval
The studies involving human participants were reviewed and approved by the Institutional Review Board of the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences, PUMC, Beijing, China (approval number: 009-2014).

Informed consent
The patients/participants provided their written informed consent to participate in this study.

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