High-Fidelity Imaging in Brain-Wide Structural Studies Using Light-Sheet Microscopy

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https://doi.org/10.1523/ENEURO.0124-18.2018

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Abstract

Light-sheet microscopy (LSM) has proven a useful tool in neuroscience to image whole brains with high frame rates at cellular resolution and, in combination with tissue clearing methods, is often employed to reconstruct the cyto-architecture over the intact mouse brain. Inherently to LSM, however, residual opaque objects, always present to some extent even in extremely well optically cleared samples, cause stripe artifacts, which, in the best case, severely affect image homogeneity and, in the worst case, completely obscure features of interest. Here, demonstrating two example applications in intact optically cleared mouse brains, we report how Bessel beams reduce streaking artifacts and produce high-fidelity structural data for the brain-wide morphology of neuronal and vascular networks. We found that a third of the imaged volume of the brain was affected by strong striated image intensity inhomogeneity and, furthermore, a significant amount of information content lost with Gaussian illumination was accessible when interrogated with Bessel beams. In conclusion, Bessel beams produce high-fidelity structural data of improved image homogeneity and might significantly relax demands placed on the automated tools to count, trace, or segment fluorescent features of interest.

Key words: light-sheet microscopy; structural imaging; whole-brain imaging; mouse; vascular/neuronal networks

Introduction

The brain is an immensely complex entity in which structure and function are intricately correlated and best elucidated on an organ-wide scale at cellular resolution. One technique, which is particularly suited for whole-brain investigations, is light-sheet microscopy (LSM; Sieden-
topf and Zsigmondy, 1902) due to its intrinsic optical sectioning capabilities, fast acquisition rates, and low photobleaching. In LSM, fluorescence is excited in a thin sheet of excitation light that coincides with the focal plane of a perpendicularly placed detection objective (Huisken et al., 2004). By combining advanced tissue clearing methods (Richardson and Lichtman, 2015; Silvestri et al., 2016; Tainaka et al., 2016) with LSM, neuronal and vascular cyto-architecture can be reconstructed over cm-sized samples like the entire mouse brain (Dodd et al., 2007; Susaki et al., 2014; Pan et al., 2016) for quantitative structural studies.

Despite its intrinsic advantages, the very nature of uncoupled, perpendicular optical pathways for fluorescence excitation and detection entails a different set of drawbacks unique to LSM. Previously published studies (Chen et al., 2016) have shown that refractive heterogeneities, always present to some extent even in extremely well optically cleared samples, lead to a loss of spatial resolution and a concomitant degradation in sensitivity and contrast. Particular to LSM are dark shadows that appear whenever the fluorescence-exciting light sheet is interrupted by scattering or absorbing obstacles in the form of bubbles, dust or other refractive index mismatches. At best, these dark shadows severely affect image homogeneity; at worst, they completely obscure any feature of interest in their path. Considering the increasingly large dataset sizes routinely produced in high-throughput LSM, high demands are placed on the automated tools to count, trace, or segment the fluorescent features of interest. Consequently, background uniformity and indeed high-fidelity imaging are paramount to facilitate the extraction of meaningful insights from terabytes of data, such as cell counts or segmentation of axons or blood vessels.

Previously published work to alleviate the problem of shadowing on the microscope system side includes pivoting the light sheet rapidly over a few degrees (Huisken and Stainier, 2007), a method incompatible with confocal line detection (Baumgart and Kubitscheck, 2012). Employing double-sided illumination (Dodd et al., 2007), a necessity when imaging intact mouse brains due to their sheer size, is not capable to improve striping since the distal light sheet cannot be made to coincide with the proximal one after having passed through the majority of the sample. Other published attempts to tackle streaky artifacts are based on post-processing, for example, in the frequency domain using nonsubsampled contourlet transform methods (Liang et al., 2016). Although successfully demonstrated, this and other algorithms are often computationally intensive and therefore neither compatible with in vivo imaging where the correction is needed at run time nor with structural imaging where terabyte-sized datasets are routinely produced in automated high-throughput microscopes.

Aiming for an optical solution to streaking artifacts, here, we apply Bessel beams (Durnin et al., 1987) to LSM to investigate biological samples for the high-fidelity interrogation of their structure. Bessel beams have been widely studied in various optical imaging methods as their propagation invariant, diffraction-free properties make them highly attractive, for example, to extend the useful field of view or depth of field (Lorenc et al., 2014; Lu et al., 2017). The “self-healing” properties of Bessel beams, on the other hand, properties enabled by the energy stored in the concentric rings replenishing the central lobe if it encounters a partial obstruction (Bouchal et al., 1998; Garcés-Chávez et al., 2002), make them robust to imaging in scattering media (Fahrbach et al., 2010). A penalty has to be paid, however, for the self-healing capabilities of Bessel beams. The on-axis irradiance in Bessel beams is lower compared to peak Gaussian beams of the same power due to the energy stored in the outer rings of the Bessel beam. Additionally the outer rings can generate out-of-focus fluorescence which means that, in general, images acquired with a Bessel beam exhibit lower contrast and inferior optical sectioning compared to Gaussian illumination, though confocal line detection (Fahrbach and Rohrbach, 2012) can be applied to ameliorate signal to background. A Bessel beam’s central core can be extremely narrow without being subject to diffraction (McGloin and Dholakia, 2005), leading to the application of Bessel beams to LSM geared toward isotropic resolution (Planchon et al., 2011; Gao et al., 2014) or studies of the scattering properties of the sample and the self-reconstructing properties of the Bessel beam itself (Fahrbach et al., 2010, 2013a). Finally, two groups have published technological development concerning Bessel beams applied to LSM (Zhang et al., 2014; Zhao et al., 2016).

Here, we report on the application of Bessel beams in LSM to investigate more specific biological samples in two typical applications, structural imaging of neurons and vasculature in the whole mouse brain. Using a direct comparative analysis between Bessel and Gaussian illumination, we provide supporting evidence and quantification of artifacts introduced by Gaussian illumination and further demonstrate that Bessel beams provide superior image homogeneity and indeed reveal structural information lost when applying standard illumination.

**Materials and Methods**

**Animals**

Transgenic mouse strains showing distinct fluorescent labeling where used for the experiments shown: Thy1-GFP-M with a sparse labeling across the entire encephalon Feng et al., 2000 ($N = 2$); B6N.Cg-Ssttm2.1(cre)U1/J × B6.Cg-
Perfusion protocol

Brain vasculature labeling of a male adult Thy1-GFP-M mouse was performed using the staining protocol described in Tsai et al. (2009), but replacing fluorescein-albumin with tetramethylrhodamine-albumin to avoid spectral overlap with the GFP expressed by neurons. After deep anesthesia with isoflurane inhalation, the mouse was transcardially perfused with 20–30 ml of 0.01 M of PBS solution (pH 7.6) and then with 60 ml of 4% (w/v) paraformaldehyde (PFA) in PBS. This was followed by perfusion with 10 ml of a fluorescent gel perfusate, with the body of the mouse tilted by 30°, head down, to ensure that the large surface vessels remained filled with the gel. The body of the mouse was submerged in ice water, with the heart clamped, to rapidly cool and solidify the gel as the final portion of the gel perfusate was pushed through. The brain was carefully extracted to avoid damage to pial vessels after 30 min of cooling and incubated overnight in 4% PFA in PBS at 4°C. The day after the brain was rinsed three times in PBS.

All experimental protocols were designed in accordance with Italian laws and were approved by the Italian Ministry of Health (authorization n. 790/2016-PR).
Amira Voltex function. The Filament Editor of Amira was used to manually trace vessels segments. Automatic segmentation of vasculature stacks was performed with Fiji by first aligning and registering both stacks to each other using the rigid body modality in the StackReg plugin. A γ of 1.1 was applied to both stacks over the entire image to enhance dimmer structures and an auto threshold using IsoData was applied. The Manders coefficients were calculated using the JACoP plugin (Bolte and Cordelières, 2006).

Statistical analysis

To estimate the amount of striping present in whole-brain acquisitions using Gaussian illumination, N = 10 animals were used. For the remaining structural imaging experiments, no sample size was computed since we investigated artifacts that are intrinsic to the microscopy method used (static shadow artifacts in LSM). Additionally, since the measurements are indeed whole mouse brain acquisitions, even with a sample size of N = 1 mouse, millions of images and terabytes of data were generated, which was more than enough to study the effects of streaking artifacts. As such, the results were illustrated on one dataset for each case study, that is: N = 1 for neuronal imaging and N = 1 for vasculature imaging. Each whole-brain tomography was repeated twice in immediate succession. Once using Gaussian illumination and then again using Bessel beam illumination. There is no biological replication since only N = 1 mouse brain was used for each comparison. There is no technical replication since each tomography was only made once. Outliers do not apply to this case/are non-existent.

**Gaussian and Bessel beams**

The typical shape for laser radiation propagating in the free space is the Gaussian beam, which takes the name from the shape of the transverse intensity profile. The Gaussian beam is composed by the superposition of plane waves with different inclinations, with an angular distribution (the so-called “angular spectrum”; Goodman, 2005) peaked on the propagation axis, with (again) a Gaussian distribution (Goodman, 2005). Since most of the energy is carried by the waves propagating along the axis direction, the presence of any scattering or absorbing particle along the beam results in a dark shadow (Fig. 1A).

Bessel beams are instead the result of the linear superposition of plane waves arranged on the surface of a cone (Fig. 1B), giving rise to a transverse intensity profile in the form of a Bessel function (Durnin et al., 1987). The peculiar angular spectrum of these beams confers their well-known self-healing property (Bouchal et al., 1998), as scattering or absorbing particles project only a conical shadow, and afterward the beam propagates unhindered (Fig. 1B). A simple and economically convenient way to generate Bessel beams is through the use of a conical lens called axicon (Indebetouw, 1989).

The Bessel beam parameters are related to the diameter of the incoming Gaussian beam and to the axicon aperture through a series of simple equations 1, 2, 3. The depth of focus δZ (Fig. 2) is given by:

\[ \delta Z = \frac{d}{\theta} \]  

where \( d \) is the radius of the incoming Gaussian beam and \( \theta \) is aperture of the cone of light that is creating the Bessel beam.

This angle is related to the refractive index \( n \) and the aperture angle of the conical lens \( \alpha \) by:

\[ \theta = (n - 1) \alpha \]  

The radius of the central core of the Bessel beam is given by:

\[ r_c = \frac{2.405\lambda}{2\pi\sin\theta} \]  

where \( \lambda \) is the wavelength of the excitation light.

A quick inspection of Equations 1, 2 readily shows another distinct feature of Bessel beams, i.e., the independence of the depth of focus and of the core radius.
Figure 3. **A**, The custom-made LSM uses double sided illumination by either Gaussian or Bessel beams. **B**, Measured PSFs for the lateral and axial direction for Gaussian (red) and Bessel illumination (cyan) using fluorescent beads. The FWHMs of the PSF are reported in the table for Gaussian and Bessel illumination, respectively. **C**, PSFs for Gaussian and Bessel illumination. **D**, Longitudinal beam profile. **E**, Transversal profile for the Gaussian (red) and Bessel beam (cyan). **F**, Beam width $\omega(z)$ of the Gaussian beam extracted from profile shown in **F**. Red line indicates fit to hyperbolic function. The beam waist $\omega_0$, the Rayleigh range $Z_R$, and the beam NA were extracted from the fit. Bottom, Table of all beam parameters. * indicates theoretically derived values.
Indeed, $d$ and $\alpha$ can be properly engineered to obtain beams with tiny central core that propagate for very long distances. This is in contrast with the conventional Gaussian beams, where the depth of focus and the beam waist are always directly proportional (Saleh et al., 1991). Bessel beams therefore allow the creation of thin and long sheets of light in LSM (Fahrbach et al., 2010). On the downside, the outer rings of the Bessel beam introduce out-of-focus excitation (see projection of a Bessel beam in Fig. 3E) that reduces the optical sectioning capabilities of the microscope. However, this problem can be slightly mitigated by means of confocal line detection (Fahrbach and Rohrbach, 2012) or more significantly by the use of sectioned Bessel beams (Fahrbach et al., 2013b).

Results

Custom LSM for structural studies of whole optically cleared mouse brains

A custom-made LSM, specifically designed for the imaging of whole optically cleared mouse brains (Müllenbroich et al., 2015), was functionally expanded to incorporate Bessel beam illumination (Fig. 3, dashed box) and...
used for all structural imaging experiments. The microscope was equipped with an alternative excitation light path using two flip mirrors that directed the light toward an axicon (AX252-A, $\alpha = 2^\circ$, Thorlabs) which created the Bessel beam by superimposing plane waves whose wave vectors lie on a cone (Indebetouw, 1989). The Bessel beam was filtered in a Fourier plane with a circular spatial filter to eliminate any residual Gaussian contribution. The Gaussian or Bessel beam, respectively, was split by a polarizing beam splitter and subsequently scanned by two galvo mirrors (6220H, Cambridge Technology) to create a light sheet that illuminated each brain half from its respective side. The excitation objectives (Plan Fluor EPI, 10x, 0.3NA, WD 17.5 mm, Nikon), covered with a protective coverslip, projected the light sheet into the focal plane of the perpendicularly placed detection objective (XLPLN10XSVMP, 10x, 0.6NA, WD 8 mm, Olympus, see inset of Fig. 3A Detection) specifically designed for immersion in high-refractive index media and featured a correction collar for the refractive index of the immersion solution, ranging from 1.33 to 1.52. A tube lens formed an image onto the sensor of a fast sCMOS camera (Orca Flash4.0 v2.0, Hamamatsu) whose line-by-line readout was synchronized to each step of the galvo mirrors to achieve confocal line detection. Appropriate bandpass filters were used to reject excitation light. For vasculature imaging, excitation was $\lambda = 561$ nm and an acousto-optic tunable filter (AOTFnC-400.650-TN, AA Opto-Electronic) was used to regulate laser power. The samples were place in a quartz cuvette (3/Q/15/TW, Starna Scientific) containing the immersion medium ($n = 1.45$, 63% TDE in PBS) and placed in a custom-made chamber filled with the same immersion medium. The samples were mounted on a high-accuracy, motorized x-, y-, z-, $\Theta$-stage (M-122.2DD and M-116.DG, Physik Instrumente) which allowed free 3D motion and rotation. The microscope was controlled via custom software written in LabVIEW 2012 (National Instruments) using the Murmex library (Distrio, Amsterdam, The Netherlands).

The optical performance of the microscope was quantified by determining the FWHM in the radial and axial directions (Fig. 3B) of the point-spread function for

![Figure 5](image-url)
Gaussian and Bessel beam modality, respectively (Fig. 3C). The lateral and axial FWHMs were 1.56 and 7.83 μm for the Gaussian beam, respectively, whereas those values were only slightly larger for Bessel beam illumination (1.77 and 8.47 μm, respectively). In LSM, lateral resolution is a standard Airy function determined solely by the wavelength and the NA of the detection objective, which explains the good agreement in lateral resolution between Gaussian and Bessel beam illumination.

The longitudinal excitation beam profiles for Gauss and Bessel are shown in Figure 3D and their transversal projections, extracted from the center of those images, are presented in Figure 3E. These projections along the detection axis, not to be confused with beam cross sections, explain the similar axial resolution between Gaussian and Bessel beam illumination.

For the Gaussian beam, a confocal parameter $b$ of 1.06 mm and a light-sheet thickness of 15 mm (FWHM) where extracted from the longitudinal profile. For Bessel beam illumination, the theoretically calculated self-reconstruction length was 1.7 mm, whereas the FWHM of the central lobe was determined at $\sim 1.2$ μm; however, the actual thickness of the light sheet, capable of exciting fluorescence, is considerably larger taking the Bessel beam side lobes into account. Indeed, Bessel beams provide higher sectioning capabilities only when coupled with structured illumination or non-linear excitation (Planchnon et al., 2011). All optical values are summarized in the table at the bottom of Figure 3.

**Streaking artifacts severely affect image homogeneity**

Here, we present the effects of streaking artifacts in structural imaging aimed at obtaining cellular-resolution maps of the anatomy over the intact optically cleared mouse brain in two case studies, firstly targeting neurons and secondly, targeting vasculature. It is apparent that in all images obtained using Gaussian illumination image homogeneity is strongly affected and dark shadows obscure microscopic anatomic features of interest. Notably those same identical features remain clearly visible when using Bessel beam illumination.

Figure 4 summarizes shadowing artifacts caused by Gaussian illumination in neuronal imaging demonstrated on axial sections of intact mouse brains (Fig. 4A–C) using a custom-made LSM (Fig. 3). Note, that each half of the intact brain is illuminated from its respective side (yellow arrows). The insets illustrate various causes of striping artefacts, including absorption by bright structures, refraction by bubbles on the brain surface, progressive attenuation of the excitation light and incomplete clearing. Substantial dark horizontal shadows traverse each brain half and completely obscure any features in their path. Using spatial filtering in Fourier space, an estimate of striping was obtained in the parallel and perpendicular
(control) direction with regard to illumination. Using whole-brain acquisitions from \( n = 8446 \) stitched slices in \( N = 10 \) animals, \( 16.4 \pm 6.3\% \) of the entire brain volume were obscured by stripes (peak of Gaussian fit, error is SD). This represents a conservative estimate since images used for analysis were downsampled to a pixel size of \( 10.4 \mu m \) to reduce computing time. To illustrate a typical variation within one single brain, Figure 4E shows the evolution of striping in axial whole-brain sections with depth throughout the intact brain.

To analyze striping in more detail, we next looked at an axial slab encompassing the entire brain of an intact Thy1-GFP-M mouse over a depth of \( 400 \mu m \) (stitched from image stacks with step size \( 2 \mu m \)). A maximum intensity projection of \( 20 \mu m \) imaged with Gaussian illumination (Fig. 5A) evidences again dark horizontal shadows which are further illustrated in an inset detailing the hippocampus. The yellow arrows mark the bidirectional illumination. By contrast, the same area acquired with Bessel beam illumination (Fig. 5B, inset) shows improved image homogeneity and the absence of strong shadowing. To quantify the extent of the area affected by strong image inhomogeneity, we calculated the line profiles obtained over the entire height of the image for Gauss and Bessel illumination, respectively (Fig. 5C) and further binarized their absolute difference (Fig. 4E) with respect to a user-selected threshold to obtain a pattern similar to a bar code. By superimposing this bar pattern to the original image (Fig. 5F) the percentage of 2D area affected by streaking inhomogeneity was estimated. Averaged over the slab and using a threshold \( 5\% \), we calculated that \( 37.5 \pm 3.1\% \) (error is SD) of the images were affected by streaking. The percentage of brain area affected by streaking as a function of the chosen threshold is presented in Figure 5D.

**Streaking artifacts obscure microscopic features of interest**

The effects of streaking artifacts on vasculature imaging are summarized in Figure 6 where an axial section of mouse brain vasculature illuminated with a Gaussian beam is shown (Fig. 6A). The white box in the olfactory bulb marks the position of details shown in Figure 6B,C for Gaussian and Bessel illumination, respectively (Movies 1 and 2). The red box marks the position corresponding to the isometric view along yz and xz illustrated in Figure 6F for Gaussian illumination whereas the cyan box is depicted in isometric view using Bessel beam illumination in Figure 6G. Although the whole-brain dataset appears to be of high quality, strong shadows in the Gaussian case completely obscure even large vessels that remain visible when illuminated with a Bessel beam (Movies 5 and 6). Using an automated segmentation based on simple thresholding (Fig. 6D,E; Movies 3 and 4; Fig. 6H,I, 3D projections; Movies 7 and 8), the Manders coefficients were averaged throughout the stack and are reported in Figure 6J. Note, that values range from 0 to 1 and express the fraction of intensity in the Gaussian channel that is located in pixels where there is non-zero intensity in the Bessel channel and vice versa. Throughout the depth of the stack the fraction of total intensity in the Bessel channel located in pixels of non-zero intensity in the Gaussian channel was \( 0.62 \pm 0.02 \), whereas the corresponding value for the Gaussian channel was \( 0.87 \pm 0.01 \) (\( p < 0.0001 \), paired \( t \) test, \( n = 39 \), error is SEM). Broadly speaking, this signifies that while \( 87\% \) of the image content present in the Gaussian channel was also present in the Bessel channel only \( 62\% \) of the image content present in the Bessel channel had corresponding content in the Gaussian channel.

**Discussion**

A particular interest in the neuroscience community is to map quantitative data of the whole mouse brain onto common atlases, a task for which LSM is particularly well suited once the mouse brain has been appropriately rendered transparent. Due to the high frame rates obtainable with LSM, whole mouse brain datasets now routinely comprise several terabytes, a size which demands automated tools (Frasconi et al., 2014; Peng et al., 2017) to count, trace, or segment features of interest either to obtain cyto-architectonic information over a mouse brain-wide scale or in the emerging field of digital 3D histology (Torres et al., 2014) to provide automated interpretation of images used for quantitative diagnosis (Bucur et al., 2015). Isolating fluorescent features of interest in a heterogeneous background places higher computational de-
mands on the algorithms used, often with concurrent increase in computation time and complexity of the pa-
parameters to be tuned. A recent study showed that very simple algorithms like global thresholding or high pass
filtering require uniform background intensity and fail to segment simple fluorescent forms like cell nuclei when a
striated background simulating muscle fibers is added to the image (Chitalia et al., 2016). As the complexity of the
fluorescent feature increases, so do the demands on the algorithms tasked to isolate them. For instance, neuron
tracing is a fundamental tool to understand neuronal mor-
phology and function, however, the accurate segmenta-
tion of neurons is to date a challenging task due to their
often complex arborization and the varying quality of
microscope images (Acciai et al., 2016).

Another recent study compared automated segmenta-
tion of a simple synthetic interrupted tube with progres-
sively added salt and pepper noise by a range of
published algorithm and their failure to accurately trace
this simulated neurite at noise levels of five percentage
(Liu et al., 2016). In Figure 5, we show how a threshold of
5% leads to more than a third of the image encompassing
half a mouse brain to be affected by a striated back-
ground, putting at risk hours of microscope acquisition
time, days of data post-processing and weeks of sample
preparation. In Figure 6, we have shown that, due to
shadowing, image content, especially of finer vessels il-
luminated with a Gaussian beam, can drop to little above
40% compared to data acquired with Bessel beam illu-
mination, again jeopardizing entire terabyte-sized data-
sets. By contrast, the quality of the datasets obtained with
Bessel beam illumination allowed, for example, the auto-
imated segmentation of blood vessels by simple thresh-
holding in areas completely obscured by shadows using
Gaussian illumination.

The self-regenerating abilities of Bessel beams are pro-
vided by the optical power in the concentric rings, which
can reconstruct the original beam profile if the central lobe
encounters any obstruction. This property makes Bessel beams attractive to counteract the various causes of
striping artefacts as presented in Figure 4. Perfecting the
degree of optical clearing in itself is not a universal solu-
tion to improved image quality since other mechanism are
also at play such as absorption by bright structures of the
brain itself or refraction by bubbles. However, the self-
healing properties of Bessel beams have to be paid with a
reduction in axial sectioning. In our setup, Bessel beam
illumination suffered from poorer axial sectioning (Fig. 3E)
caused by the excitation of fluorescence by the optical
power stored in the concentric rings. Optical sectioning
could have been improved by employing segmented Bes-
sel beams (Fahrbach et al., 2013b), or by using non-linear

**Movie 2.** Raw data of the vasculature of a Thy1-GFP-M mouse
labelled with tetramethylrhodamine-albumin imaged with Bes-
sel beam illumination. See Figure 6C for details. [View online]

**Movie 3.** Segmented data of the vasculature of a Thy1-GFP-M
mouse labelled with tetramethylrhodamine-albumin imaged with
Gaussian illumination. Automated segmentation was based on
simple thresholding. See Figure 6D for details. [View online]
excitation strategies (Planchon et al., 2011). These strategies will however introduce further complexity to the optical system and increase the overall cost of the apparatus. In any case, our standard Bessel-beam light-sheet microscope is capable of producing much more uniform images at the cost of an axial resolution only slightly worse than its Gaussian counterpart.

In conclusion, we have illustrated how streaky artefacts introduced by Gaussian illumination can adversely affect and even nullify data extracted from LSM images. We compared the performance of Gaussian and Bessel beam illumination in structural studies, covering brain-wide morphology of neuronal and vascular networks in optically cleared mouse brains. We have found that over a third of the tested volume was adversely affected by illumination inhomogeneity and that, in the worst case, microscopic features of interest are irrecoverably lost. We have shown how the use of Bessel beams can provide an optical solution to correct for these artifacts on the microscope system side and allow for high-fidelity imaging in LSM. The results presented here redefine the quality standard for quantitative measurements in LSM with a single neuron sensitivity that opens up a new class of experimental studies.
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