INTRODUCTION

Organotypic brain slice cultures are useful for studying interactions among brain cell types, including neurons and glial cells, and for examining the structure and function of neural circuits, which are preserved similar to in vivo when this method is used. Three main methods are used for organotypic slice cultures: (a) roller-tube cultures, (b) interface cultures, and (c) collagen gel cultures. The most common brain slice culture method is interface culture in which brain slices are placed on semiporous polytetrafluoroethylene (PTFE) membrane inserts that are resting in medium-containing tissue culture wells. Our laboratory uses PTFE membranes placed...
on O-shaped donut plate inserts for interface brain slice cultures (Figure 1A).²

Live imaging of slice cultures enables monitoring of cellular dynamics such as migration, growth of cellular processes, and cell–cell interactions. Such live imaging has classically been performed by either observing cultured slices from above (upright microscope) or from below (inverted microscope).³⁻⁷

Live imaging of slice cultures has been widely conducted using upright microscopes equipped with the water immersion lenses or the long distance dry objective lenses.⁴⁻⁶ Meanwhile, when inverted microscopes are used for live imaging, the observation of deep regions of slice cultures is difficult because the distance between the objective lens and the slice usually surpasses the range of the lens’s focal length. In addition, the low transparency of the PTFE membrane worsens the situation. Thus, culture membranes to improve the use of inverted microscopes for live imaging of slice cultures are needed.

Here, we report a novel method for live imaging of slice cultures using a collagen membrane primarily consisting of collagen type I, which has a high cellular affinity. Collagen membranes are thinner and have a higher optical transparency than PTFE membranes, making them suitable for live imaging of slice cultures using an inverted microscope.

**FIGURE 1** A, Representative images of PTFE and collagen membranes. Membranes were placed in donut plates for slice cultures. B, Representative images of PTFE and collagen membranes using a scanning electron microscopy at 3000× (upper) and 30 000× (lower) magnification. C, Thickness of PTFE and collagen membranes. *** P < .001 vs PTFE membranes; Student’s t test, n = 9 membranes. Data represent mean ± SD. D, The transmittance of PTFE and collagen membranes from 400 to 800 nm. *** P < .001 vs PTFE membranes; two-way repeated-measures ANOVA followed by Tukey’s test, n = 9 membranes. Data represent mean ± SD.
2 METHODS

2.1 Animals

Experiments were performed with the approval of the animal ethics committee at the University of Tokyo (approval number: P29-10) and according to the University of Tokyo's guidelines for the care and use of the laboratory animals. Experiments were conducted using postnatal day 6 (P6) C57BL/6J and CX3CR1<sup>GFP/+</sup> pups. The mice were housed under controlled temperatures and light schedule (23-25°C and a 12-hour light/dark cycle) and given unlimited access to food and water.

2.2 Slice culture

The preparation and maintenance of slice cultures, including culture media, were performed as previously described. Horizontal entorhinohippocampal slices (400-μm-thick) were placed on PTFE membrane filters with a published pore size of 0.45 μm (JHWP02500; Millipore) or collagen membrane filters (Kanto Chemical Co., Inc) which were then placed into donut plates (Hazai-Ya). Collagen membranes are mainly made of bovine collagen type I. White support film was used to prevent collagen membranes from curling up. Collagen membranes can be directly purchased from Kanto Chemical Co., Inc.

2.3 Scanning electron microscopy

Collagen membranes were fixed in 2.5% glutaraldehyde and dehydrated using increasing concentrations of ethanol. PTFE and collagen membranes were mounted on carbon tape, sputter coated with platinum-palladium, and examined under a scanning electron microscope (S-4500; Hitachi) using a 10 kV accelerating voltage.

2.4 Membrane thickness

PTFE and collagen membranes were hydrated with Milli-Q water. After hydration, membrane thickness was measured using a micrometer (CLM1-15QM).

2.5 Optical transparency

PTFE and collagen membranes were dissected and hydrated with Milli-Q water. Membranes were placed on glass slides (S0990490; FIGURE 2 A, Representative images of the entorhinohippocampal slice cultures at 7 d in vitro (DIV) immunostained for caspase-3. Nuclei were traced with Hoechst. B, The density of caspase-3-positive cells cultured on PTFE and collagen membranes. P > .05 vs PTFE membranes; Student’s t test, n = 6 slices. Data represent mean ± SD. C, Representative images of the hippocampal slice cultures at 14 DIV immunostained for NeuN and Iba1 and DNA counterstained with Hoechst. D, E, The density of NeuN-positive cells D, and Iba1-positive cells E, at 14 DIV P > .05 vs PTFE membranes; Student’s t test, n = 5-7 slices. Data represent mean ± SD. F, Representative images of hippocampal slice cultures at 14 DIV immunostained for Iba1. G, Cumulative distribution of total microglial process length at 14 DIV P > .05 vs PTFE membranes; Kolmogorov-Smirnov test, n = 62 processes, from 7 to 8 cells, from 3 slices.
2.6 | Immunohistochemistry

Cultured slices were fixed in 4% paraformaldehyde at 4°C for 24 hours. Next, the slices were permeabilized and blocked for 1 hour using 0.3% Triton X-100 with 10% goat serum in PBS. Primary antibody staining was performed using mouse anti-NeuN (1:1000; MAB3777; Merck Millipore), rabbit anti-Iba1 (1:1000; 019-19 741; FUJIFILM Wako Pure Chemical Co.), rabbit anti-caspase-3 (1:400; 9662; CST), and guinea pig anti-Iba1 (1:500; 324 006; Synaptic System, Göttingen, Land Niedersachsen, Germany) followed by Alexa Fluor 488-, 594-, and 647-conjugated secondary antibody staining (1:500; Thermo Fisher). Finally, the samples were embedded in PermaFluor (Thermo Fisher). Images of immunostained samples were obtained using the SpinsRF10 (Olympus) confocal system with 10× (NA = 0.40) and 40× (NA = 0.95) objectives (Figure 2A-E, Figure 3). Z-series images were collected at 2.0 μm steps and stacked for 6 slices for caspase-positive cell density analysis (Figure 2A,B) and 0.5 μm steps and stacked for 21 slices for microglia and neuron density analysis (Figure 2C-E). The stacked images were analyzed using ImageJ software (NIH, Bethesda, MD, USA). Single-plane images were shown at each depth in Figure 3.

2.7 | Analysis of microglial morphology and dynamics

For the analysis of microglial morphology and dynamics, slice culture specimens were observed using the SpinSR10 (Olympus) confocal system with a 30× (NA = 1.05) objective. Z-series images were collected at 1.0 μm steps and stacked for 21 slices. For time-lapse imaging, slice cultures were maintained in a humidified chamber under 37°C and 5% CO₂ conditions and the images were obtained at 30 second intervals. The stacked images were analyzed using ImageJ software (NIH). For the quantification of total processes (main processes and short branches) length, confocal images were obtained after immunohistochemistry (Figure 2F,G). For the quantification of density and dynamics of short branches (Figure 4), confocal images were obtained in time-lapse imaging.

3 | RESULTS

The membrane microstructure of both PTFE and collagen membranes was investigated using scanning electron microscopy (Figure 1B). We observed that both PTFE and collagen membranes were semiporous, allowing culture medium to infiltrate into the slice cultures. Collagen membranes were also significantly thinner than PTFE membranes (Figure 1C). These properties contributed to the significantly higher transparency of collagen versus PTFE membranes that was observed at all wavelengths (between 400 and 800 nm; Figure 1D).

To determine whether collagen membranes would be suitable for brain slice cultures, mouse entorhinohippocampal slices were cultured on both PTFE and collagen membranes. First, cell death was examined using caspase-3 immunostaining at 7 days in vitro (DIV) (Figure 2A). There were no significant differences in the density of caspase-3 staining between slice cultures on PTFE or collagen membranes (Figure 2B). Next, the density of NeuN-positive neurons and Iba1-positive microglia was examined at 14 DIV (Figure 2C). There were no significant differences in the density of neurons or microglia between slice cultures on PTFE or collagen membranes (Figure 2D,E).
To evaluate the influence of membrane type on microglial morphology, we examined the length of total microglial process at 14 DIV (Figure 2F,G). Total microglial processes were defined as main process and short branch. To analyze total microglial processes, the main process of microglia was first determined. The main process of microglia was defined as a process that directly emanated from the soma and possessed the longest length from the emanating point to the tip compared to the other processes that emanated from the same soma. Then, the branches emanated from main processes were defined as short branches. There was not a significant difference in total microglial process length between slice cultures on PTFE or collagen membranes (Figure 2G). These results suggest that the cellular conditions in slice cultures on collagen membranes were comparable to those on PTFE membranes and collagen membranes can be used to culture entorhinohippocampal slices.

Finally, live imaging of slice cultures on PTFE or collagen membranes was conducted using an inverted confocal microscope. To visualize the morphology of microglial cells, slice cultures were prepared from the brains of CX3CR1^{GFP/+} mice. Microglia in these mice have been engineered to express green fluorescent protein (GFP). The short branches that emanated from the main process of microglia in slice cultures on collagen membranes were presented in Figure 3A. The density of visible short branches on PTFE membranes was higher than PTFE membranes at 0 seconds (Figure 4B). The movements of these short branches were observed and presented in Figure 4C-E.
were followed during live imaging (Figure 4C, arrows). Though the
density of visible short branches were significantly lower in slice
cultures on PTFE membranes than those on collagen membranes,
the dynamics of visible short branches were comparable between
collagen and PTFE membrane conditions (Figure 4D). Additionally,
we found that microglia in slice cultures on collagen membranes
continuously extended and retracted the short branches (Figure 4E).
Thus, collagen membranes allowed the observation of changes in
small cellular structures in deep regions of cultured slices during live
imaging using an inverted microscope.

4 | DISCUSSION

In this study, we examined whether collagen membranes would be
useful for live imaging of cellular structures in slice cultures using an
inverted microscope. We found that cells in slices cultured on col-
genous spines or organelles may be detected in live imaging using a
membrane determines which substrates can diffuse into the slice for
their survival, it can be predicted that PTFE membranes with a pore
size of 0.45 μm allow the penetration of nutrients such as salts, glu-
cose, amino acids in essential media and growth factors, adhesion
molecules, hormones, lipids, and minerals in serum.

From our scanning electron microscopy images (Figure 1B), it
was difficult to determine the actual pore size in collagen mem-
because the collagen fibers had a mesh-like structure and
highly overlapped. A previous study reported that the average pore
sizes of collagen membrane used in our experiments were about
1-2 μm using scanning electron microscopy, which is larger than
that of the PTFE membrane (0.45 μm). If the pore size of collagen
membrane is actually 1-2 μm, the nutrients required for cell survival
can be diffused into slices on the collagen membrane. Though the
possibility that the pore size of the collagen membrane is smaller
than 1-2 μm cannot be excluded, our findings suggested that the
degree of cell death (Figure 2B) and the density of neurons and mi-
croglia (Figure 2D,E) were comparable between slice cultures on
PTFE membranes and collagen membranes. Thus, it is predicted
that the necessary nutrients were supplied to slice cultures on col-
gen membrane.

Collagen membranes enabled live imaging of short branches of
microglia. It has been reported that the morphology of microglia at
the top and bottom surfaces of slice cultures is different from that
observed in vivo, while microglia in the center region are morpho-
logically similar to microglia in vivo. Therefore, the center region of
the slice culture should be used for microglial morphology studies.
In the present study, we found that the use of collagen membranes
was suitable for this purpose. While we only studied the kinetics
of microglial short branches, other cellular microstructures such as
neuronal spines or organelles may be detected in live imaging using
this method.

Collagen is a major extracellular matrix protein that supports
cellular development, differentiation, and morphology in vivo and
has long been used as a cell culture dish coating material. Neurons
cultured on collagen gel-coated glass slides are known to
survive well. Many studies have suggested that cells cultured on
collagen gel-coated glass or plastic dishes survive better than cells
cultured on uncoated dishes. Such results suggest that collagen
membranes may have a higher affinity for cultured slices compared
to PTFE membranes. It would be interesting to develop slice culture
membranes coated with extracellular matrices other than collagen,
such as laminin or proteoglycan, or a mixture of these matrices.
Membranes that are tailored made to best suit the specific aims of each
experiment would expand the application of slice cultures.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
AO conducted the experiments, analyzed the experimental data, and
wrote the manuscript. TA helped prepare slice cultures. RK designed
and planned the project and wrote the manuscript. YI discussed the
results and commented on the manuscript.

ANIMAL STUDIES
All experiments were performed with the approval of the animal ex-
periment ethics committee at the University of Tokyo and according
to the University of Tokyo’s guidelines for the care and use of labora-
tory animals.

DATA REPOSITORY
The data that support the findings of this study are available in the
supplementary material of this article.

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