Combining cell-encapsulation technique and axon guidance for cell transplantation therapy

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Short title: Combining encapsulation and axon guidance

Abstract

In cell transplantation therapy for the treatment of neurodegenerative disorders, encapsulation of implanted cells in a semipermeable membrane is a promising approach to protect the implanted cells from host immune rejection and inhibit the invasion of tumor into surrounding tissue if the implanted cells form a tumor after transplantation. However, implanted neurons isolated by capsules could not build connections with host neurons, preventing the implanted neurons from responding to stimuli from host neurons. In the present study, we focused on the passage of neurites and axons navigated by axon guidance molecules through membrane pores to enable encapsulated neurons and host neurons to form connections. The type of matrix coated on membranes and the pore size of the membranes greatly affected the successful passage of PC12 neurites through membrane pores. PC12 neurites preferably passed through collagen-coated membranes with pores greater than 0.8 μm in diameter, but the neurites did not pass through albumin- or fibronectin-coated membranes or membranes with pores less than 0.1 μm in diameter. We could navigate the direction of commissural neural axon extensions by utilizing the axon guidance molecules secreted from floor plate and make guided axons pass through the membrane pores. These results suggest the feasibility of building connections between encapsulated neurons and host neurons by encapsulating the implanted neurons and axon guidance molecules, which attract the axons of host neurons into the capsule, in the porous membranes with suitable pore size and matrix coating.

Key words: Cell transplantation therapy, Encapsulation, Axon guidance, Commissural neuron, Porous Membrane

Introduction

Cell transplantation to replace lost neurons is a new approach for the treatment of neurodegenerative disorders [1]. Research in the field of Parkinson's disease, which is characterized by a reduction in striatal dopamine associated with the relatively selective loss of nigro-striatal dopaminergic neurons, has spearheaded the exploitation of this approach [2-5]. Some studies have reported a beneficial response after the transplantation of human fetal dopaminergic neurons into patients with Parkinson's disease. However, the feasibility of this therapy is limited by the severe shortage of human fetal tissue and ethical problems. Development of an efficient, safe, and ethically acceptable source of cells for transplantation is also a major issue for the treatment of other neurological disorders such as Alzheimer's disease, Huntington's disease, and diabetes insipidus [6-8]. Recently, neurons induced from stem cells are expected to be a valid alternative cell source. *In vitro* studies have shown that various types of neurons could be generated from stem cells, including human neural stem cells, human embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells, and used for the development of stem cell-based cell transplantation therapy [9-14]. However, grafts

of cells derived from stem cells can be contaminated with residual pluripotent cell types, leading to tumor formation in the host [15].

The other major obstacle for cell transplantation therapy is the rejection of implanted cells by the host immune system [16, 17]. Encapsulation of implanted cells in a semipermeable membrane has been examined to avoid this obstacle [18, 19]. The selectively permeable nature of the polymer membrane permits bidirectional access of low molecular weight compounds, including the inward diffusion of oxygen and other vital nutrients and the outward diffusion of cell products such as dopamine and insulin. The membrane restricts the passage of elements of the host immune system including immunocompetent cells, antibodies, and complement proteins, thereby preventing host rejection of the encapsulated cells. In addition, cell encapsulation can prevent the invasion of tumors into surrounding tissue if implanted cells form a tumor after transplantation [20]. These benefits of encapsulation can enable the use of stem cell-derived neurons as a cell source for transplantation. Date et al. and Aebischer et al. demonstrated that dopamine-secreting PC12 cells, a catecholaminergic cell line derived from rat pheochromocytoma, encapsulated in a hollow fiber and implanted into the striatum of Parkinsonian model monkeys and adult guinea pigs, respectively, stably provided dopamine to the striatum [21, 22]. Although the encapsulation of implanted cells has some benefits, conventional capsules such as hollow fibers and alginic acid hydrogel prevent the formation of synaptic connections between implanted neurons and host neurons due to the small

pore size required to prevent the passage of antibodies and complement proteins [23, 24]. Since neurons communicate with one another via synapses *in vivo*, formation of synaptic connections between implanted neurons and host neurons is important for implanted neurons to work effectively in response to the stimuli from host neurons.

To enable encapsulated neurons and host neurons to form connections, we propose the encapsulation of implanted neurons and axon guidance molecules, which can attract the axons of host neurons into the capsule, in membranes with pores sized to allow the passage of axons in the central nervous system with a typical 1-μm diameter but prevent the passage of immunocompetent cells with a diameter of about 6 - 15 μm (Fig. 1). Because human cells will be used in future clinical settings, we expect that a membrane that can prevent the passage of host immunocompetent cells, which play a major role in the rejection of implanted allogenic cells, is sufficient. When these encapsulated neurons are implanted into the host brain, axons of host neurons can be attracted toward the capsule, supporting the formation of synaptic connections between encapsulated neurons and host neurons. In the present study, we focused on the passage of neurites and axons navigated by guidance molecules through membrane pores as a proof-of-concept demonstration of the feasibility of our approach. First, we examined the effects of matrix coated on the membrane and the pore size of the membrane on the passage of neurites through membrane pores by using PC12 cells. Second, we navigated the direction of axon extension of commissural neurons by using axon guidance

molecules secreted from the floor plate, allowing guided axons to pass through the membrane pores.

Materials and Methods

Passage of PC12 neurites through porous membranes

To examine the effect of different matrix-coated membranes on the passage of neurites through membrane pores, polycarbonate Nuclepore® membranes with 5-μm diameter pores (thickness, 10 μm; Whatman Inc., Florham Park, NJ) were immersed overnight at 37°C in PBS solutions containing: 0.005% polyornithine (weight-average molecular weight: 30~70 kDa; Sigma, St. Louis, MO); 10 μg/ml mouse laminin (BD Biosciences, Bedford, MA); 10 μg/ml bovine plasma fibronectin (Invitrogen, Carlsbad, CA); 10 μg/ml rat tail collagen Type I (BD Biosciences); or 5% bovine serum albumin (Sigma). The membranes were then washed with PBS. To examine the effect of the membrane pore size on the passage of neurites through the pores, polycarbonate Nuclepore® membranes with a pore diameter of 0.05, 0.1, 0.4, 0.8, 1, or 3 μm (thickness, 6 - 10 μm; Whatman Inc.) were immersed in 10 μg/ml collagen solution in PBS overnight at 37°C and then washed with PBS. The presence of protein on the membrane was confirmed by using the protein staining reagent, Coomassie brilliant blue.

PC12 cells (JCRB0266, Health Science Research Resources Bank, Japan) were grown in culture dishes in RPMI 1640 medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Invitrogen), 10% horse serum (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were collected by pipetting and resuspended in culture medium. Then, the cells were plated on the various matrix-coated membranes or collagen-coated membranes with various pore sizes at a density of 6×10^4 cells/cm^2. After 5 h of incubation, 2.5S-nerve growth factor (NGF, Invitrogen) at a final concentration of 100 ng/ml was added to the culture medium, followed by cultivation for 6 days in a humidified 5% CO_2 incubator at 37°C. After 6 days, cells on membranes were fixed with a 4% paraformaldehyde solution for 15 min at 4°C and then permeabilized in methanol for 15 min at –20°C. After blocking for 1 h with 2% skimmed milk solution, the cells were incubated with rabbit anti-β-tubulin type III (TuJ1; Babco, Richmond, CA, USA) (diluted to 1:600 with 2% skimmed milk solution) overnight at 4°C. The samples were washed with 0.05% polyoxyethylene sorbitan monolaurate (Tween20) three times and then incubated with FITC-labeled rabbit IgG (Jackson, West Grove, PA) (diluted to 1:100 with 2% skimmed milk solution) for 2 h at room temperature. After washing three times using 0.05% Tween20, cell bodies were removed from the upper membrane surface by wiping with a cell scraper (Iwaki, Tokyo, Japan). Then, stained neurites were visualized with a IX71 fluorescent microscope (Olympus, Tokyo, Japan) that was focused on the underside of membranes. For quantification, the total area of stained neurites was measured by using ImageJ

software. Five independent experiments were performed. To compare the different types of matrix, we calculated the ratio of the total area of stained neurites on each membrane to that on the collagen-coated membrane. For the examination of pore size, the total area of stained neurites on each membrane was normalized by dividing the total area of stained neurites on each membrane by the total pore area of each membrane, and the results were expressed as the ratio of the normalized total area of stained neurites on each membrane to that on the membrane with 3-μm diameter pores. For statistical evaluation, experimental groups were compared by analyzing the variance followed by *t*-tests with Welch's correction.

Passage of axons navigated by guidance molecules through porous membranes

The experimental procedure is schematically shown in Fig. 2. Nylon membrane with 60-μm diameter pores and 50-μm thicknesses (2 _ 2 mm; Millipore, Tokyo, Japan) was glued vertically to the polystyrene cell culture dishes and then washed with ethanol. Spinal cords were isolated from E13 Wistar rat embryos by dissection in L15 medium (Invitrogen) [25]. Spinal cords were cut at the roof plate and flattened down in an open book configuration. About 400 _ 400 μm pieces of dorsal spinal cord and floor plate explants were harvested from opened spinal cords by using tungsten needles in L15 medium containing 5% horse serum. Dorsal spinal cord and floor plate explants were placed on the nylon membrane-attached substrates facing each other across the nylon membrane.

Then, substrates were embedded in three-dimensional collagen gels and cultivated in 50% F12 medium (Invitrogen), 39% Opti-MEM (Invitrogen) and 5% horse serum supplemented with 40 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37°C. After a 24-h incubation period, cells were observed with an IX71 phase-contrast microscope. The above experimental protocol was approved by the AIST's ethical committee for animal experiment.

Results and Discussion

Effect of matrix coated on the porous membrane on passage of neurites through membrane pores

Matrix coated on the membrane is thought to greatly influence the passage of axons through membrane pores. We examined the passage of extending neurites through various matrix-coated membranes by using PC12 cells as a model. When PC12 cells are exposed to NGF, they differentiate into sympathetic neuron-like cells, extending long neuronal-like processes [26]. After attaching the PC12 cells to the upper side of various matrix-coated membranes and subsequently stimulating the neurite outgrowth by the addition of the NGF, neurites stained with a neural marker, TuJ1, on the underside of the membranes (i.e., neurites passing through the membrane pores) were observed. Representative fluorescent images and a more quantitative assessment of neurites from PC12 cells

extending through the pores of various matrix-coated membranes are shown in Fig. 3 and Fig. 4, respectively. Remarkable passage of neurites was found on polyornithine-, collagen-, or laminin-coated membrane, in contrast to the uncoated membrane (Fig. 3 a-d). Our results are consistent with previous reports that cationic polymers, such as polyethylenimine, polyornithine, or polylysines, and collagen- or laminin-coated substrates support the attachment of neural cells and the extension of neurites [27-29]. On the other hand, no passage of neurites was observed on the fibronectin- or albumin-coated membranes (Fig. 3 e, f). These results are closely related to the cell-adhesion behavior on these membranes (Fig.5). Cell adhesion on fibronectin-coated membranes was significantly inferior to adhesion on polyornithine-, collagen- or laminin-coated membranes. Albumin-coated surfaces are well-known to be resistant to cell adhesion, and the PC12 cells did not attach to the albumin-coated membranes [30]. Thus, the type of matrix coated on the membrane significantly affects the passage of neurites, and collagen is the most effective coating for promoting the passage of PC12 neurites (Fig. 4). It is worth mentioning that the pore diameter of the membranes may be decreased in the range of several ten nanometers after the coating of matrix due to the adsorption of matrix onto the pore walls in all experiments [31].

Effect of pore size of the porous membrane on the passage of neurites through membrane pores

Pore size is also thought to influence the passage of axons through membrane pores. Therefore, PC12 cells were cultured on collagen-coated membranes with various pore sizes. Representative fluorescent images and a more quantitative assessment of neurites from PC12 cells extending through membranes with various pore sizes are shown in Fig. 6 and Fig. 7, respectively. Remarkable passage of neurites occurred on membranes with pore sizes larger than 0.8 μm, whereas the passage of neurites hardly occurred with pore sizes smaller than 0.1 μm (Fig. 6 a, b, d, e, f). A small but significant passage was found on the membranes with 0.4-μm pores (Fig. 6 c). No significant difference in the passage of neurites was seen between membranes with 0.8-, 1-, and 3-μm pores, and a decreased passage of neurites was observed between the membranes with 0.4- and 0.8-μm pores; thus, pore size did not influence the passage of PC12 axons above 0.8-μm pore (Fig. 7). Membranes with 0.8- to 3-μm diameter pores can prohibit the passage of host immunocompetent cells, but cannot inhibit the passage of antibodies and complement proteins [32, 33]. It has been accepted that the transplantation of allogenic cells causes the activation of cellular immunity [34]. On the other hand, humoral immunity including antibodies and complement proteins is thought to play a major role in the rejection of implanted xenogenic cells [35]. Hence, we expect that a membrane that can prevent the passage of host immunocompetent cells is sufficient, because human cells will be used in future clinical settings. Although the effective type of matrix and minimum pore size for the passage of axons may depend on the type of neurons, a suitable matrix and pore size can

be identified by performing examinations in the same manner as in the present study.

Passage of commissural axons navigated by guidance molecules through membrane pores

During spinal cord development, commissural neurons in the dorsal spinal cord extend axons toward the floor plate by the actions of netrin-1 and Shh secreted from the floor plate cells (Fig. 8 a) [36, 37]. This attraction of axons at a distance by diffusible chemoattractants *in vivo* was reproduced *in vitro* [38]. When dorsal spinal cord explants containing commissural neurons were cultured with floor plate explants in the collagen gel matrix, commissural axons, as axon bundles, extended toward the floor plate explants (Fig. 8 b, c), while no axon bundles were observed in the absence of floor plate explants (Fig. 8 d). This co-culture system of dorsal spinal cord and floor plate explants was applied as a model for displaying the passage of axons navigated by axon guidance molecules through the membrane. Because commissural neurons extend axons as a bundled structure, we selected the porous membrane with large-diameter pores (60 μm) for this experiment. Porous membrane was placed between dorsal spinal cord explants and floor plate explants, and these explants were cultured in the collagen gel matrix (Fig. 2). As shown in Fig. 8 e, f, axon bundles of commissural neurons in dorsal spinal cord explants were attracted toward the floor plate explant, passing through the membrane pores. Some molecular families such as Slits, Semaphorins, BMPs, and Wnts, in addition to Netrins and Hedgehogs, have also been identified as diffusible axon

guidance molecules, which attract or repel the axons of various types of neurons at a distance [39, 40]. These diffusible axon guidance molecules are also good candidates for controlling the direction of axon extension, making axons pass through the membrane pores. In addition to these guidance molecules, growth factors and extracellular matrix molecules affect axon guidance and extension. Thus, optimization of the combination of regulating molecules for each neural subtype along with the type of membrane is necessary to create an effective axon control system for transplantation.

Conclusions

Here, we focused on the passage of neurites and axons navigated by axon guidance molecules through membrane pores. Our studies of PC12 cells cultured on porous membranes show that the type of matrix coated on the membrane and the pore size of the membrane greatly affect the successful passage of PC12 neurites through membrane pores. Moreover, we could navigate the direction of commissural neural axon extension by utilizing axon guidance molecules secreted from floor plate explants and make guided axons pass through the membrane pores. These results provide valuable information for improving capsule materials to promote connections between encapsulated neurons and host neurons. Recently, neurons induced from stem cells have attracted much attention as unlimited cell sources for cell transplantation therapy. A major concern with the use of stem cell-derived neurons is their potential for tumorigenicity. Because encapsulation restricts tumorigenesis

by physically restricting the proliferation of tumor cells, the development of cell-encapsulation techniques may contribute to the spread of stem cell-based cell transplantation therapy.

Acknowledgements

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Figure captions

Figure 1. Concept of the cell transplantation therapy utilizing the cell-encapsulation technique and axon guidance.

Figure 2. Schematic representation of the preparation method of explant co-culture separated by a porous membrane.

Figure 3. Representative fluorescent images of PC12 neurites extending through pores of (a) uncoated polycarbonate membrane, (b) polyornithine-coated membrane, (c) collagen-coated membrane, (d) laminin-coated membrane, (e) fibronectin-coated membrane, and (f) albumin-coated membrane. Scale bar, 100 μm.

Figure 4. Passage of PC12 neurites through various matrix-coated porous membranes. Results are expressed as the ratio of the total area of stained neurites on each membrane to that on collagen-coated membrane. Data are represented by the means ± SD, n=5.

Figure 5. Cell adhesion on various matrix-coated porous membranes after 5-hour cultures. Phase-contrast micrographs of PC12 cells cultured on (a) uncoated polycarbonate membrane, (b)

polyornithine-coated membrane, (c) collagen-coated membrane, (d) laminin-coated membrane, (e) fibronectin-coated membrane, and (f) albumin-coated membrane. Scale bar, 200 μm.

Figure 6. Representative fluorescent images of PC12 neurites extending through membrane pores with (a) 0.05-μm diameter, (b) 0.1-μm diameter, (c) 0.4-μm diameter, (d) 0.8-μm diameter, (e) 1-μm diameter, and (f) 3-μm diameter. Scale bar, 100 μm.

Figure 7. Passage of PC12 neurites through porous membranes with various pore sizes. Results are expressed as the ratio of the normalized total area of stained neurites on each membrane to that on membrane with 3-μm diameter pores. Data are represented by the means ± SD, n=5. †; $p < 0.0001$, ††, †††; $p > 0.01$.

Figure 8. Passage of axons navigated by guidance molecules through porous membranes. (a) Schematic diagram of a cross-section of the vertebrate spinal cord. (b) Phase-contrast micrograph of co-culture of dorsal spinal cord explants (right side) with floor plate explants (left side). (c) Magnified view of axons extended from dorsal spinal cord explants in (b). (d) Phase-contrast micrograph of dorsal spinal cord explants alone. (e) Phase-contrast micrograph of co-culture of dorsal spinal cord explants (right side) with floor plate explants (left side) separated by a porous

membrane. The arrow shows the porous membrane. (f) Magnified view of axons passing through the membrane pores in (e). Scale bar, 200 μm.

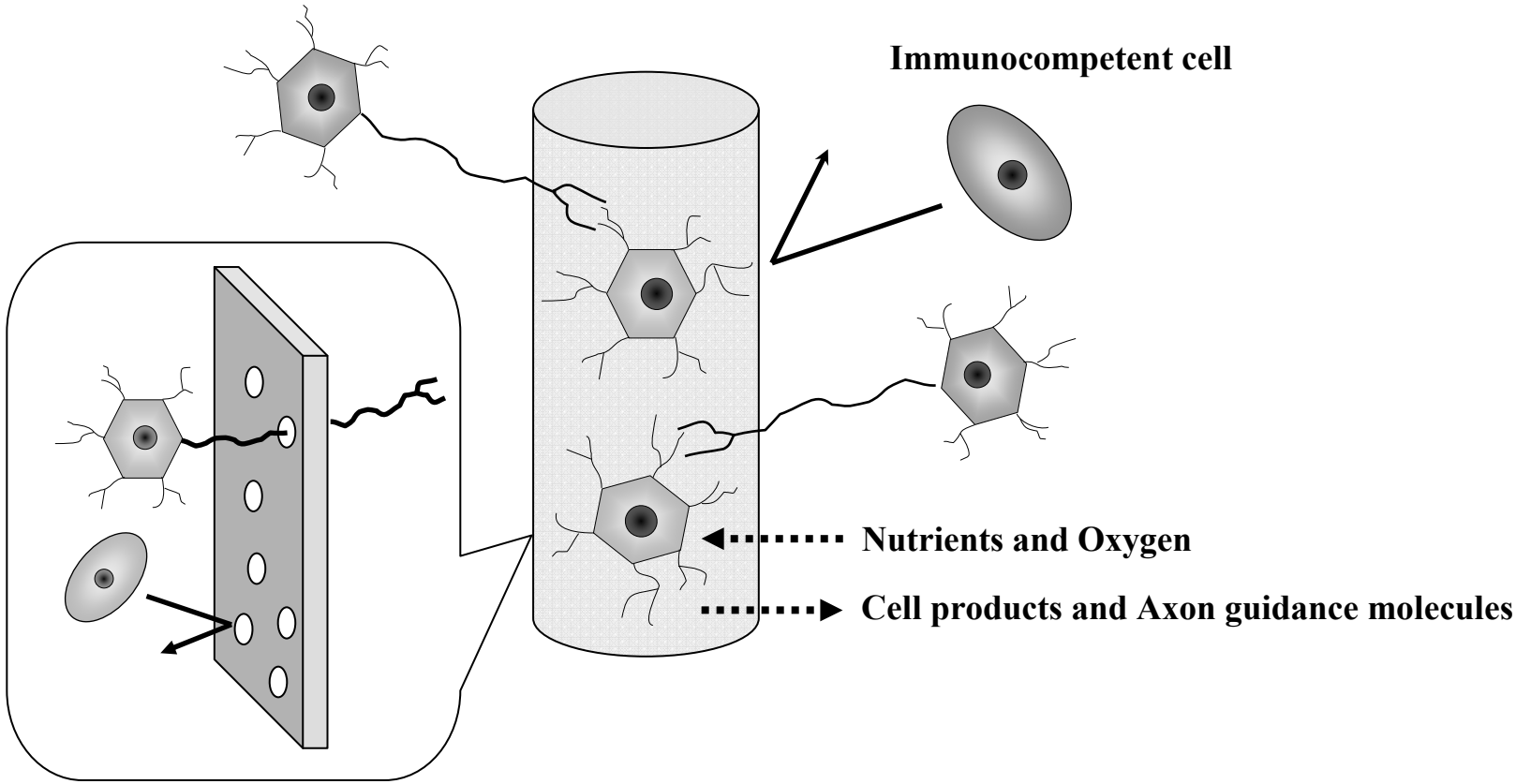


Figure 1 Yamazoe et al.

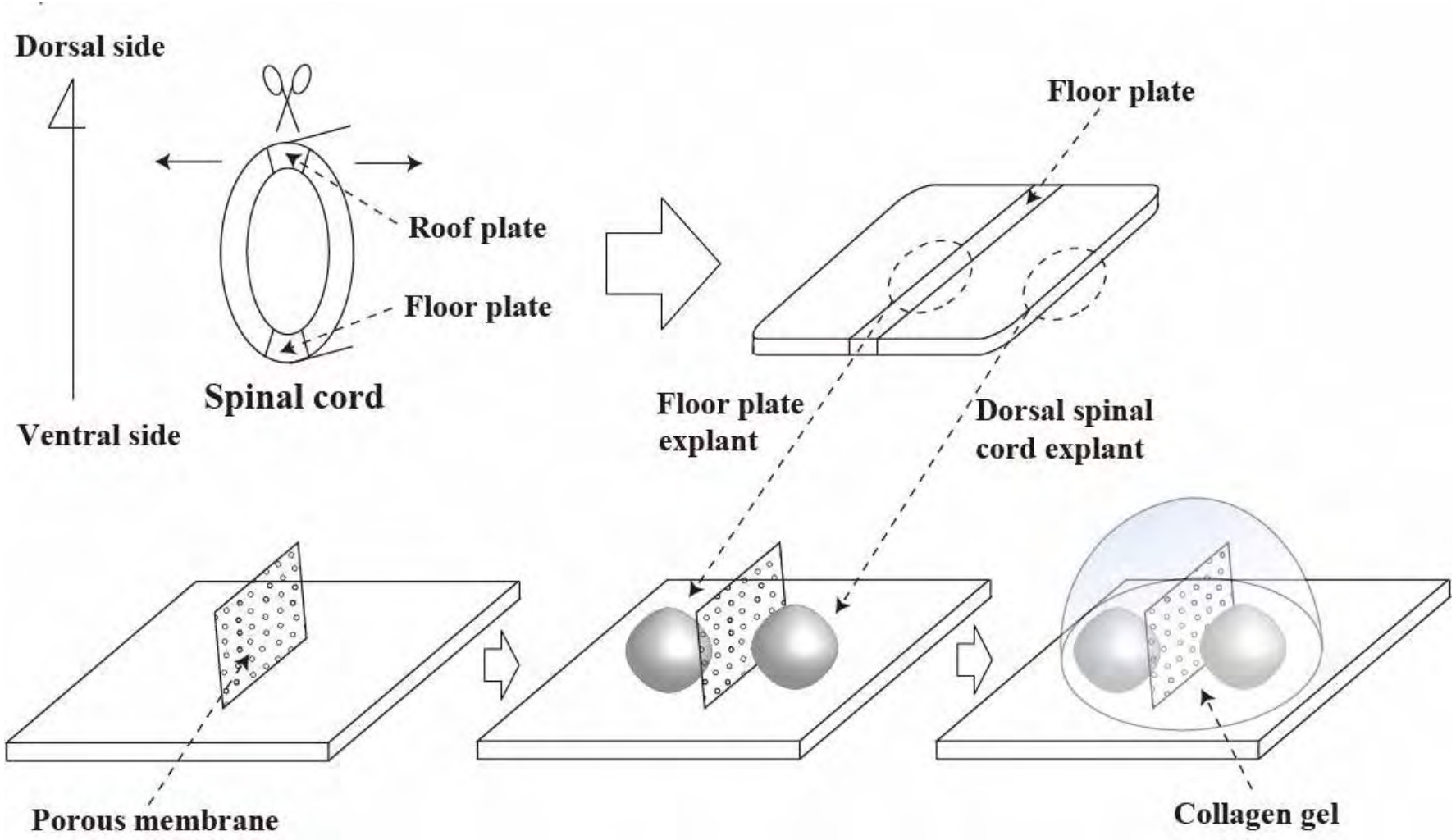
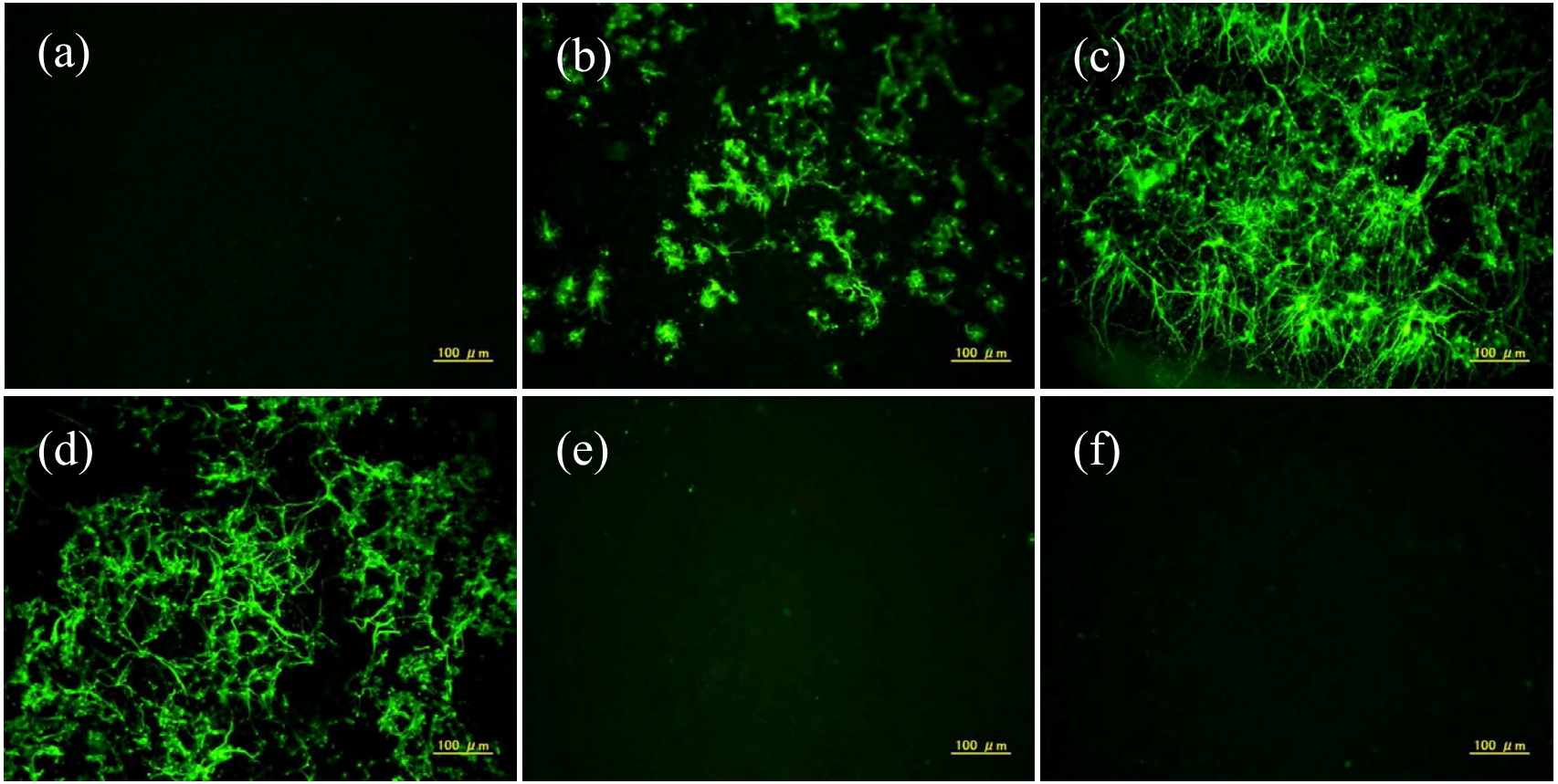


Figure 2 Yamazoe et al.



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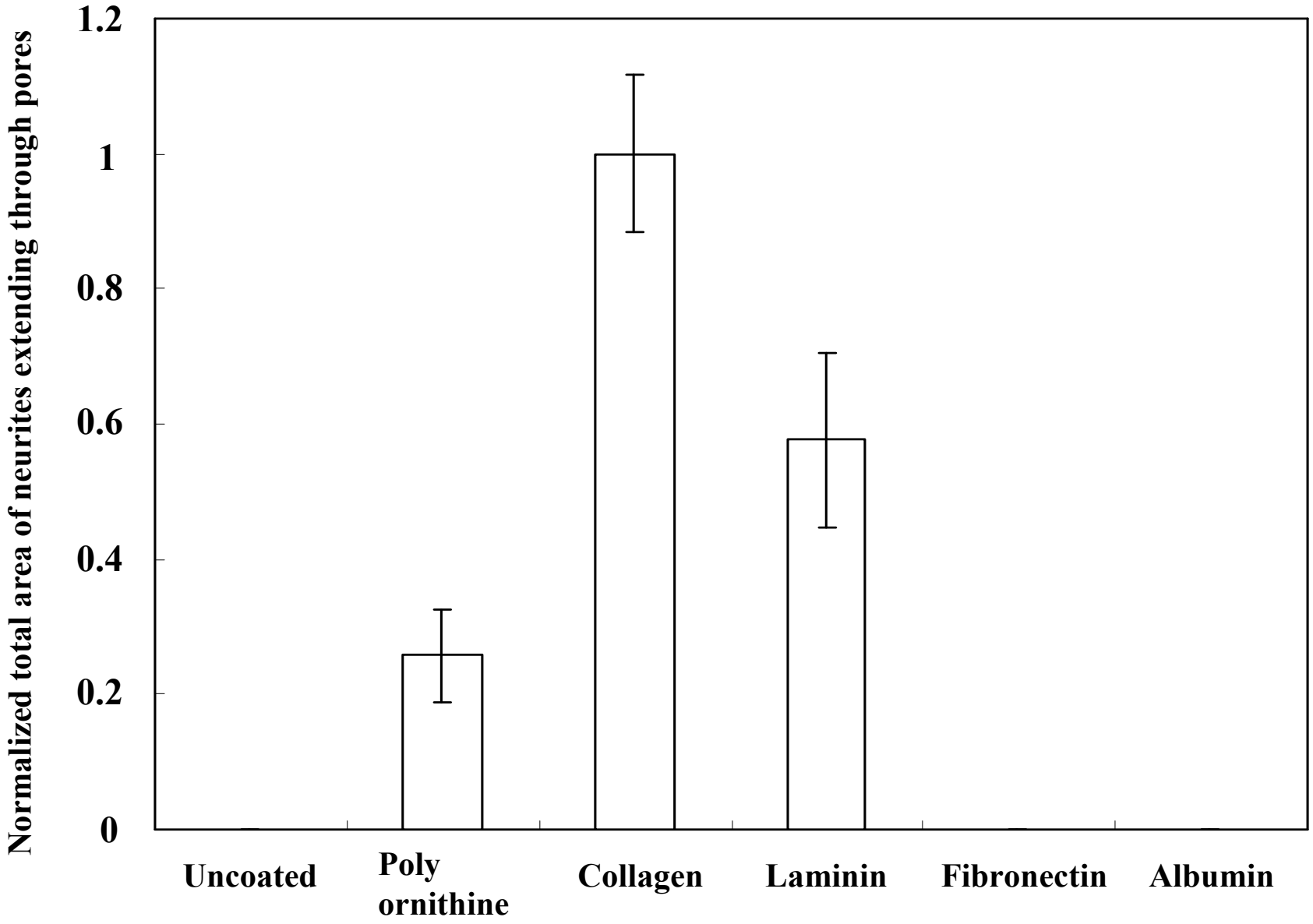

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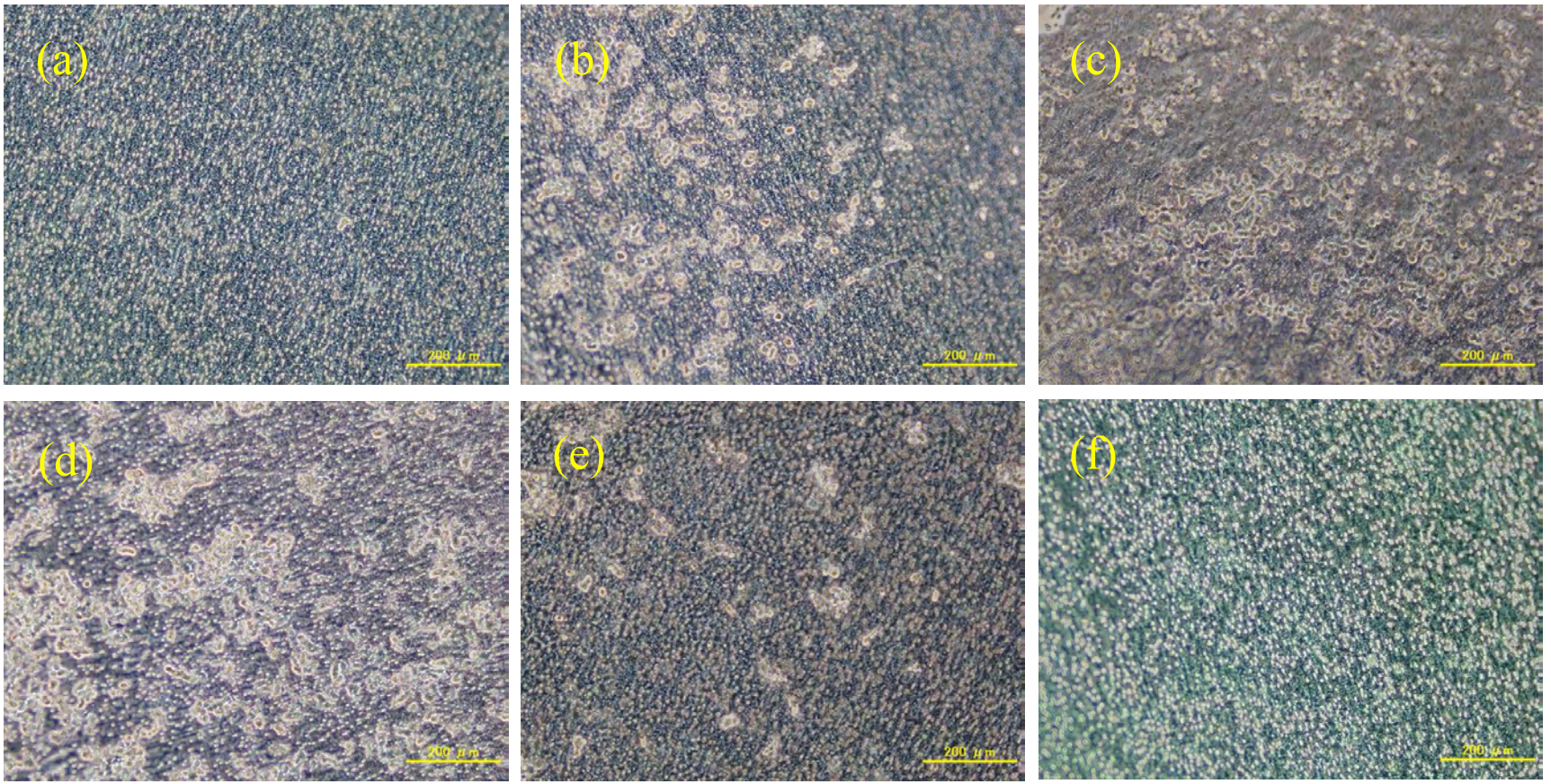

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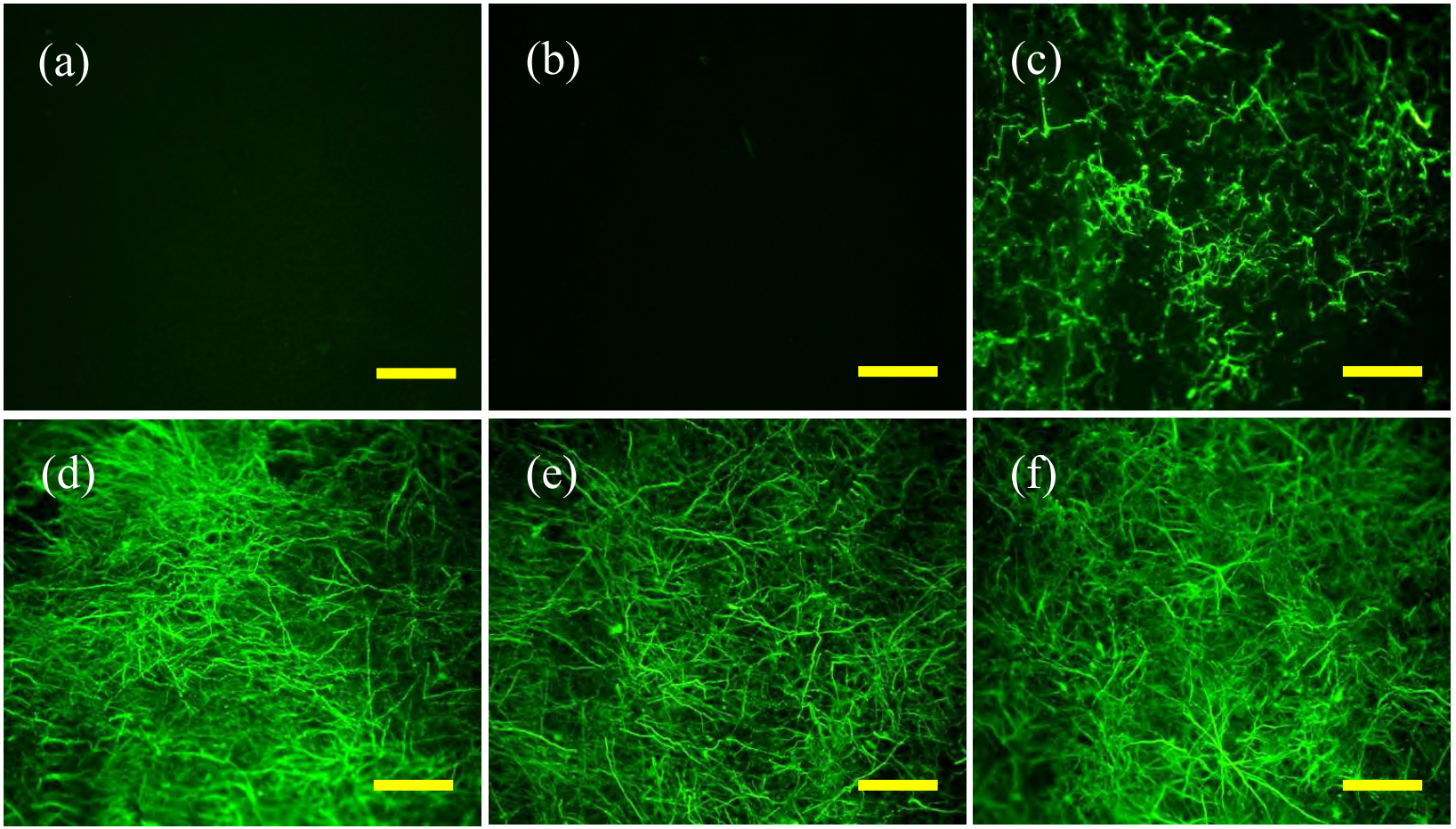

Figure 6 Yamazoe et al.

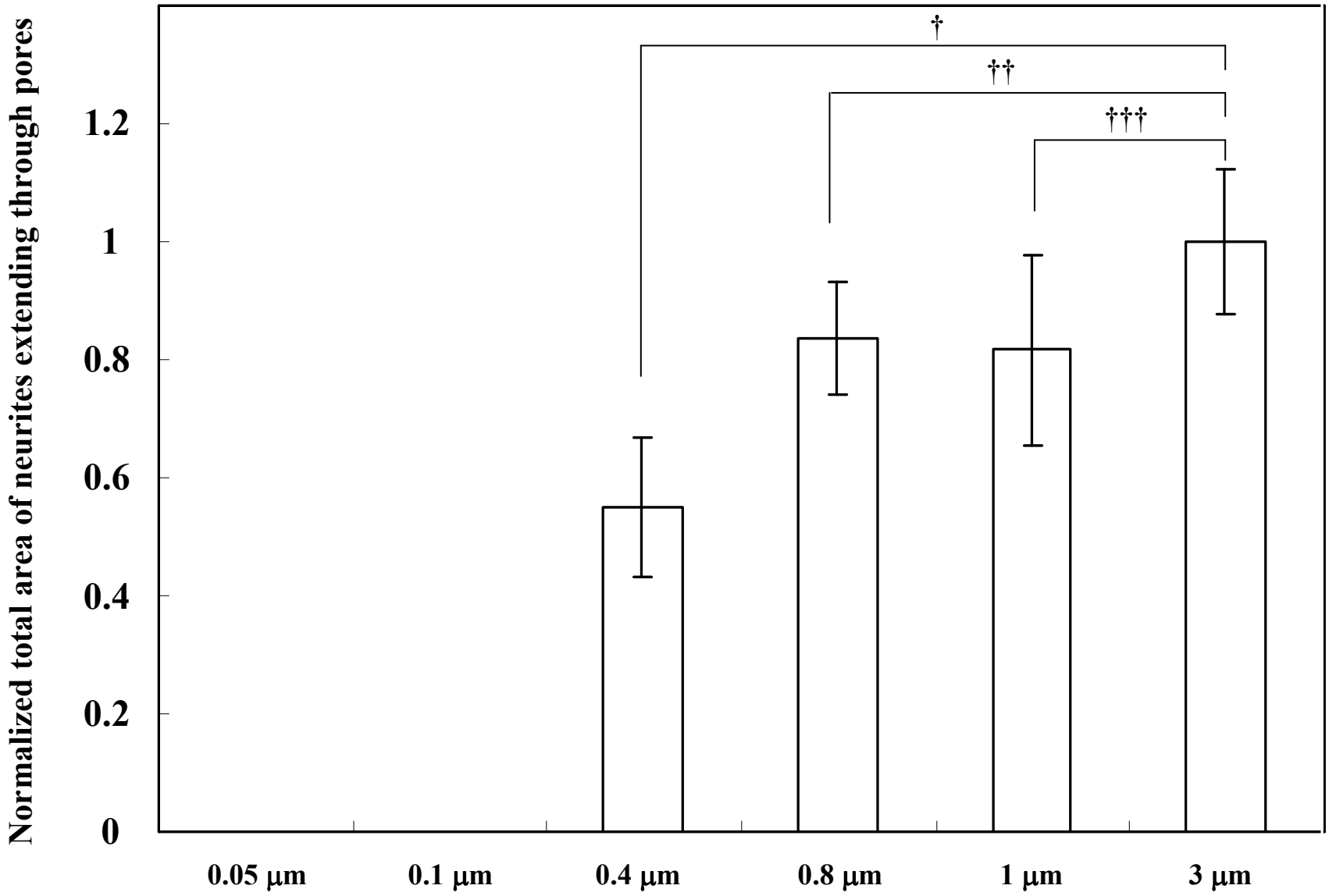

Figure 7 Yamazoe et al.

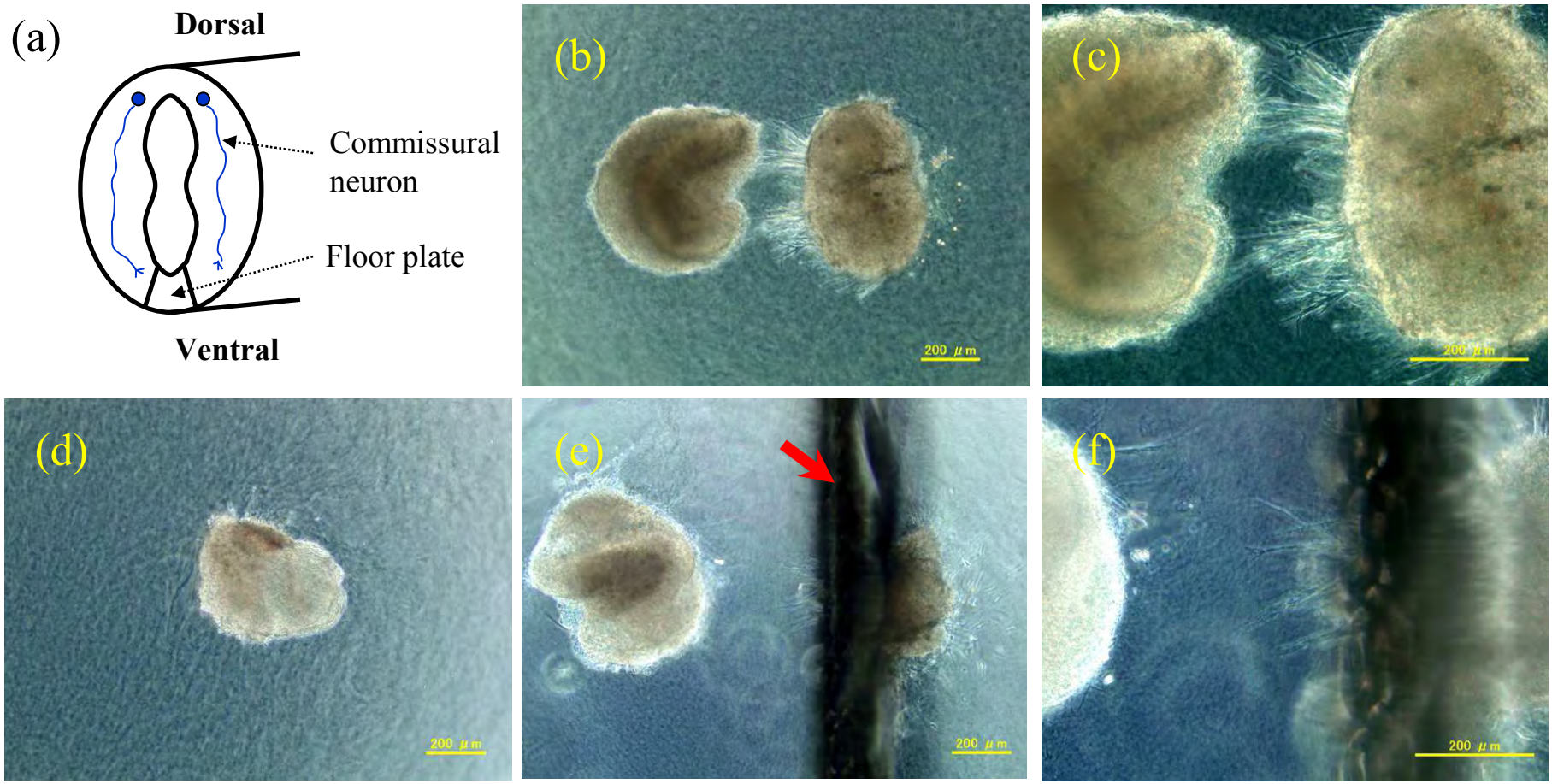

Figure 8 Yamazoe et al.