Flexible neuronal network patterning during cultivation using collagen gel photo-thermal etching

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Abstract— We developed a 3D collagen gel photo-thermal etching method using an infrared laser that precisely controls the area of cell adhesion and neurite projection by etching a small targeted section of the collagen gel. It was then possible to guide neural network formation under microscopic observation. After conventional cell seeding, we succeeded in creating isolated 3D networks while controlling (1) the number of each neural subtype (neurons, glia, and fluorescent-labeled neurons) and (2) the direction of neurite elongation. Neurons seeded on a 10-μm-thick collagen gel survived longer and projected greater numbers of neurites than neurons growing on 2D culture substrates. Intracellular Ca$^{2+}$ imaging revealed both synchronous and discordant oscillations in different neuronal populations that suggested the pattern and strength of synaptic connectivity. This photo-thermal etching technique allows for the creation of designed 3D neural networks during cultivation for use in studies of synaptic transmission, neuron–glial signaling, pathogenesis, and drug responses.

I. MATERIAL & METHODS

To fabricate collagen gel culture dishes amenable to photo-thermal etching, we first fixed indium tin oxide (ITO) glass on a 35-mm dish. The glass was then coated with a 10-μm-thick layer of 1, 2, 3, or 4 mg/ml liquid rat tail collagen (Life Technologies) and spread using a spin coater (2200 rpm for 25 s). A 1064-nm infrared laser beam was focused on the collagen gel layer on the ITO glass, causing the collagen gel at the focal point to melt. Precise etching patterns in the collagen gel were produced by moving the automated x–y stage on the microscope. Rat hippocampal neurons were cultured on collagen gels. Identification of collagen fibers, specific cell types, and synaptic connections was performed by immunofluorescence staining of aldehyde-fixed cultures. To demonstrate the control of neural subtypes within the network using collagen gel photo-thermal etching, we also cultured neurons from E18 heterozygous SD-Tg (CAG-EGFP) rat embryos, which contain both wild-type neurons and neurons expressing EGFP.

II. RESULTS AND CONCLUSION

Using photo-thermal etching of the collagen substrate, we succeeded in creating neuronal networks with controlled cell numbers and connectivity patterns. We confirmed that neurons did not grow beyond the isolated collagen gel area (and across an etched collagen gap) by microscopy and verified that all collagen fibers in the etched area were lost by immunostaining with anti-collagen type I. Even after 3 weeks in culture, neurons and neurites did not grow into the etched area. After examining cell morphology under the microscope and fluorescence microscope at 3 DIV, we isolated an astrocyte and a neuron, an EGFP(+) and a wild-type neuron from surrounding cells using photo-thermal etching. Small neural networks with controlled cell numbers and cell types (wild-type hippocampal neuron, glia, and neurons expressing EGFP) were created. To create neuronal networks with precisely defined connectivity patterns, we first selected two neurons with unconnected neurites at 2 DIV and then guided neurite elongation by controlling the cultivable area between them using photo-thermal etching. The two neurons projected neurites into a narrow collagen gel bridge (etched on both sides). Neurons appeared connected by 4 DIV.

We compared the viability and morphology of neurons growing on etched 3D collagen gels to neurons growing on 2D agarose gels. A collagen concentration of 2 mg/ml was optimal for promoting neuronal survival, neurite length, and neurite number. The superior growth on the collagen gel versus the agarose gel suggests that neuronal survival and morphology are dependent on both the flexibility of ECM and the contact area. Finally, we measured spontaneous changes in [Ca$^{2+}$], in single neurons and six-neuron networks. Neurons in 3D collagen gel cultures exhibit activity patterns characteristic of other neurons and neural networks in vitro.

We succeeded in constructing 3D neural networks containing specified numbers and types of cells and controlled the direction of neurite elongation. This technique is simple and does not require highly specialized fabrication equipment or environments. This method will be useful for the study of cell–cell interactions, synaptogenesis, and drug screening assays using induced pluripotent stem cell-derived neurons.