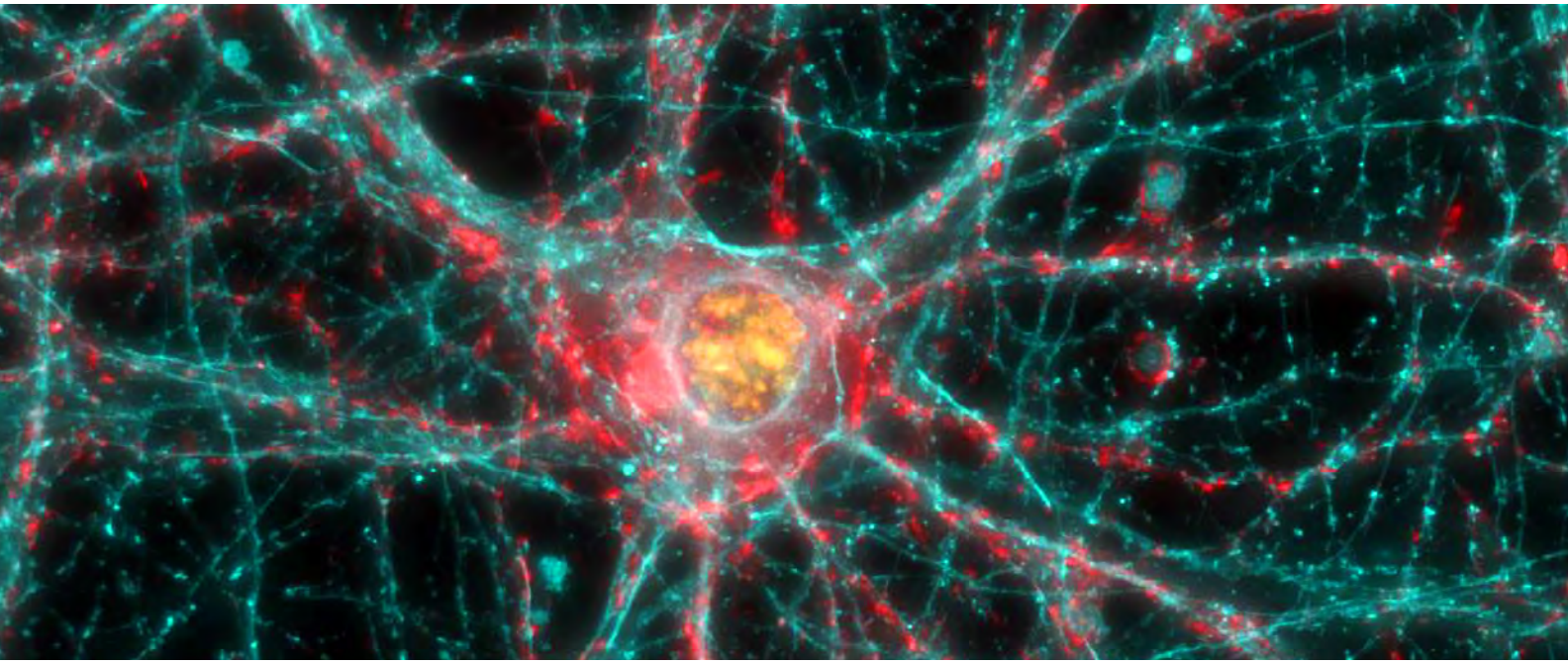

Tools for 2D and 3D neuronal cell culture

From the B-27 Plus Neuronal Culture System to fluorescent probes for cell analysis.

Traditional culture and analytical methods in the neuroscience field have long relied on rodent cell model systems to emulate the behaviors of higher mammalian neural network activity, with the aim of discovering, characterizing, and testing pharmaceuticals for safety and toxicology. These and most excitable, postmitotic cell types are typically dissociated from freshly prepared tissues or cryopreserved for later study using a variety of media and extracellular matrix formulations intended to recapitulate native or *in vivo* conditions. To this end, the commercial availability of large-scale batches of quality-controlled, cryopreserved primary cell types has helped advance the study of brain and nerve function. Also available are improved media formulations specifically developed to increase neuronal survival and enhance maturation of neuronal phenotypes in extended cultures of primary and induced pluripotent stem cell (iPSC)-derived neurons, as well as functional probes designed for use with 2D and 3D neuronal cultures.

Figure 1 (above). Visualizing a mouse cortical neuron culture. Cryopreserved Gibco™ Primary Mouse Cortical Neurons (Cat. No. A15585) were grown in culture for 3 weeks using the Gibco™ B-27™ Plus Neuronal Culture System (Cat. No. A3653401). Cells were fixed and labeled with Invitrogen™ NucBlue™ Fixed Cell ReadyProbes™ Reagent (Cat. No. R37606), anti–β-tubulin mouse monoclonal antibody (Cat. No. 32-2600) in conjunction with Invitrogen™ Alexa Fluor™ 488 goat anti–mouse IgG antibody (Cat. No. A11029), and Invitrogen™ ActinRed™ 555 ReadyProbes™ Reagent (Cat. No. R37112). Cells were mounted in Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980) and imaged on an Invitrogen™ EVOS™ FL Auto 2 Imaging System using a 60x oil immersion objective.

Advancing neuronal culture

For over 25 years, researchers have relied on Gibco™ B-27™ Supplement and Gibco™ Neurobasal™ Medium for their neuronal culture applications [1]. The original serum-free B-27 Supplement was developed for the long-term culture of rat hippocampal and cortical neurons. However, the publication history of B-27 supplements demonstrates that researchers are expanding their use far beyond these applications. Progress in the development of iPSC-derived neurons has led to the need to maintain a higher density of neurons over longer periods of time than B-27 Supplement and Neurobasal Medium were designed to support. We have applied our decades of experience with culture media to develop the Gibco™ B-27™ Plus Neuronal Culture System—a serum-free culture system that provides increased neuronal survival, accelerated neurite outgrowth, and improved electrophysiological activity and maturation of neurons.

A closer look at neuronal subtypes

The B-27 Plus Neuronal Culture System improves upon the classic culture environment through both raw material and manufacturing upgrades and minor formulation modifications. Here we look at how improved neuronal culture conditions affect subpopulations of neurons in primary neuronal cultures and the functional implications of enhanced subpopulation survival and maturity. Preparations of neurons from rodent brains contain different types of cells, including excitatory (glutamatergic) and inhibitory (GABAergic) neurons, and a subset of GABAergic interneurons that are positive for parvalbumin (PV). *In vivo*, the numbers of excitatory and inhibitory neurons are tightly regulated, and their interactions are the foundation for neural network regulation. Further, PV interneurons play a critical role in controlling synchronous activity across networks. *In vitro*, neuronal survival and robust maturation are vital to neuronal subpopulation maintenance and the formation of networked and synchronous activity in primary cortical and hippocampal cultures.

The impact of culture conditions on subpopulations of excitatory and inhibitory rat cortical neurons is shown in Figure 2, using comparisons between 28-day cultures grown in the classic B-27 Supplement and Neurobasal Medium and in the B-27 Plus system. The total numbers of neurons in each culture were determined by counting HuC/D-positive cells. Using neuronal subpopulation–specific antibodies, we further characterized these cells as glutamatergic neurons (VGLUT2-positive), GABAergic neurons (GABA-positive), and PV-positive interneurons.

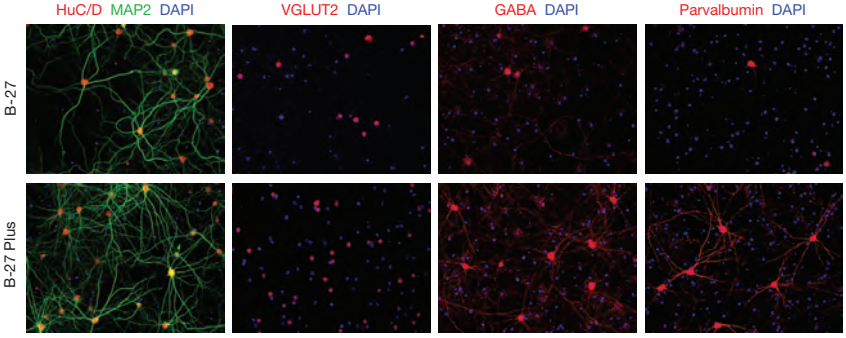

Figure 2. Defining neuronal subtypes in cultures of primary rat neurons. Rat cortical neurons were cultured for 28 days in either the classic Gibco™ B-27™ Supplement and Gibco™ Neurobasal Medium (top row) or the Gibco™ B-27™ Plus Neuronal Culture System (bottom row), and then immunostained for MAP2 (green, Cat. No. PA5-17646) and HuC/D (red, Cat. No. A21271), VGLUT2 (red, Cat. No. 42-7800), GABA (red, Cat. No. PA5-32241), or parvalbumin (red, Cat. No. PA1-933) and the corresponding Invitrogen™ Alexa Fluor™ 488 donkey anti–rabbit IgG (Cat. No. A21206) and Alexa Fluor™ 594 donkey anti–mouse IgG and anti–rabbit IgG (Cat. No. A21203, A21207) secondary antibodies. Nuclei were labeled with DAPI (blue).

Quantitative image analysis performed on the Thermo Scientific™ CellInsight™ CX5 High-Content Screening (HCS) Platform showed approximately 50% more HuC/D-positive cells in the B-27 Plus system than in the classic B-27 system. In addition, 90% of all neurons in the classic B-27 system stained positive for VGLUT2, compared with 81% in the B-27 Plus system, and 12% of the neurons in the B-27 cultures were GABA-positive neurons versus 14% in the B-27 Plus system. Strikingly, the percentage of PV-positive interneurons was less than 1% in the classic B-27 system, compared with 7% in the B-27 Plus system. These results are in line with estimates of 80% glutamatergic and 20% GABAergic for cortical neurons *in vivo*. Qualitative analysis of the MAP2, GABA, and PV staining indicates significantly more staining of developed neurites in cells cultured in the B-27 Plus system, suggesting enhanced maturation of the neurons and their subpopulations. Taken together, these data indicate significant improvements in the maintenance and maturation of neuronal subpopulations in the B-27 Plus system.

Challenges to neuronal maturation and analysis of 2D and 3D cultures

One of the principal barriers to generating useful data with neural culture systems is the length of time required for stem cell–derived and primary cultures to reach maturation for downstream functional analysis. Typically, culture medium must be refreshed 2–3 times

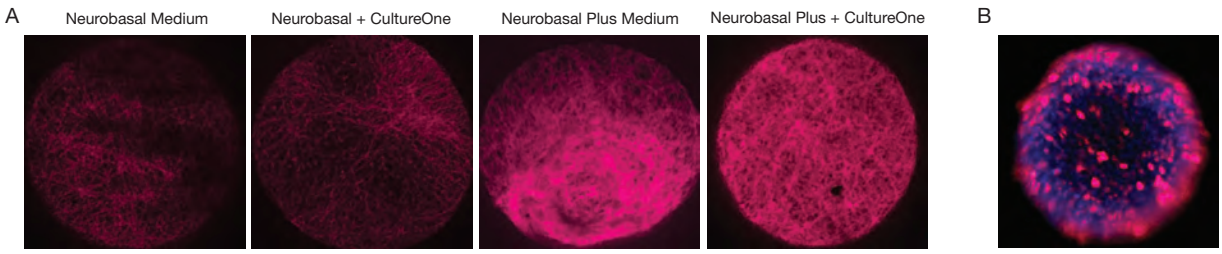

Figure 3. 3D neuronal cultures stained with Tubulin Tracker Deep Red. (A) Neuronal spheroids were cultured using Gibco™ Neurobasal™ (Cat. No. 21103049) or Gibco™ Neurobasal™ Plus (Cat. No. A3582901) Medium, with and without Gibco™ CultureOne™ Supplement (Cat. No. A3320201), in Thermo Scientific™ Nunclon™ Sphera™ 96U-Well Microplates, and then stained with Invitrogen™ Tubulin Tracker™ Deep Red reagent (Cat. No. T34077). Images were generated using a 10x objective on the Thermo Scientific™ CellInsight™ CX7 High-Content Analysis (HCA) Platform. **(B)** Neuronal spheroids were labeled with Tubulin Tracker Deep Red and Invitrogen™ NucBlue™ Live ReadyProbes™ (Cat. No. R37605) reagents. Cells were imaged in Gibco™ HBSS containing calcium and magnesium (Cat. No. 14025134), supplemented with probenecid (Cat. No. P36400). Images were generated using an Invitrogen™ EVOS™ FL Auto 2 Imaging System with a 20x objective and an Invitrogen™ EVOS™ Cy®5 Light Cube.

per week for a minimum of 2 weeks for synapses to form and stable networks to emerge in the cultures. This challenge becomes exceptionally difficult in the context of 3D cultures, where medium must be carefully removed and replaced without disturbing or accidentally aspirating the spherical organoids formed by immature progenitors. Neural stem cell (NSC)-derived cultures can take 4 weeks or more to become functionally responsive to depolarizing stimuli, and longer still to form synapses.

To address these challenges, the Neurobasal Plus culture system was specifically developed to accelerate the maturation rate of primary cultures and increase the yield of neurons that survive the long road to differentiation. Additionally, the Gibco™ CultureOne™ Supplement was designed to halt proliferation of progenitor cells and redirect them toward a neural lineage in roughly half the time of unsupplemented cultures. Culturing NSCs with the Neurobasal Plus system and CultureOne supplement resulted in improved functional responses from terminally differentiated cells that are almost entirely lacking progenitor phenotypes and greatly enriched for markers of neuronal character.

The advantages of using CultureOne Supplement are especially important in 3D NSC cultures. To form 3D neuronal spheroids, NCSs were differentiated in Neurobasal or Neurobasal Plus Medium, with and without CultureOne Supplement, for 2 weeks and then stained with Invitrogen™ Tubulin Tracker™ Deep Red reagent to label neuronal processes. This cell-permeant reagent provides a far-red– fluorescent, tubulin-selective dye that requires only a single stain-and-wash step. Because media changes and staining protocols alike are pain points for 3D culture protocols, Tubulin Tracker Deep Red reagent is an ideal cell analysis tool, eliminating the need to fix and permeabilize spheroids before analysis (in contrast to immunostaining protocols). As demonstrated by TubulinTracker Deep Red staining and imaging on the Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform, neuronal spheroids show enhanced neurite growth when grown in Neurobasal Plus Medium with CultureOne Supplement, compared with those grown in the original Neurobasal Medium without supplement (Figure 3).

Along with improvements in neuronal media, recent developments in 3D culture systems and supports have enhanced the ability to grow neurons in a matrix that more closely resembles the state of nervous system tissue in intact organisms, without requiring the use of expensive and time-consuming animal models. The neuronal spheroids shown in Figure 3 were grown on Thermo Scientific™ Nunclon™ Sphera™ Microplates, which have a surface with very low cell-attachment properties. Nunclon Sphera plates consistently promote 3D spheroid and organoid culture.

Explore 2D and 3D culture and analysis

Thermo Fisher Scientific offers a suite of culture media, cultureware, cell analysis reagents, and fluorescence instrumentation for 2D and 3D neuronal cell cultures. Learn more at **thermofisher.com/neurobasal**. ■

Reference

1. Brewer GJ, Torricelli JR, Evege EK et al. (1993) *J Neurosci Res* 35:567–576.

Product	Quantity	Cat. No.
B-27™ Plus Neuronal Culture System	1 system	A3653401
B-27™ Plus Supplement (50X)	10 mL	A3582801
CultureOne™ Supplement (100X)	5 mL	A3320201
Neurobasal™ Plus Medium	500 mL	A3582901
Nunclon™ Sphera™ 96U-Well Microplate	1 case of 8	174925
Tubulin Tracker™ Deep Red	60 slides 300 slides	T34077 T34076
GABA Polyclonal Antibody	100 µL	PA5-32241
HuC/HuD Monoclonal Antibody (clone 16A11)	100 µg	A21271
MAP2 Polyclonal Antibody	100 µL	PA5-17646
β-Tubulin Monoclonal Antibody (clone 2 28 33)	100 µg	32-2600
Parvalbumin Polyclonal Antibody	100 µg	PA1-933
VGLUT2 Polyclonal Antibody	100 µg	42-7800