HIGH CONTENT PROTOCOL

Synaptogenesis Assay

Version 1

Introduction

The Thermo Scientific™ Synaptogenesis Assay enables simultaneous detection of neuronal population, neurite, pre-synaptic vesicle, post-synaptic puncta and synapse using a fixed end-point assay based on immunofluorescence detection in cells grown on standard high-density microplates.

The molecular network between synapses controls synaptic signal transmission and plasticity and regulates neuronal growth, differentiation and death. To understand the relationship between synaptic activity and neuropathophysiology and the molecular mechanism involved in synaptogenesis and synapse regulation, the microstructure of synaptic junction has been extensively studied.[1] The modulation of neurite and synaptic structures in neurons are closely related to the pathological process of neurological diseases or in neurodevelopment.[2]

The Synaptogenesis Assay was optimized using the Thermo Scientific™ ArrayScan™ HCS Reader using the Neuronal Profiling BioApplication™ Software Module, which identifies the synapse measured by colocalization of the pre-synaptic marker with the post-synaptic marker. Thus, automated plate-handling, focusing, cell image acquisition and processing, and data analysis and management are combined in one high-content analysis (HCA) system to assay for test compounds.[3] In addition to HCS instruments, cells labeled using the reagents in this assay can be viewed and analyzed by other fluorescence microscopes.

Materials Required

Description	Recommended Product No.	Concentration / Units	Recommended Amount per 96-Well Plate
MAP2 Primary Antibody (mouse)	MA5-12826	500µL†	10-20µL
DyLight 550-Conjugated Goat Anti-Mouse Secondary Antibody	84540	1mg/mL	10-15µL
PSD95 Primary Antibody (mouse)	MA1-046	100µL†	10-20µL
DyLight 488-Conjugated Goat Anti-Mouse Secondary Antibody	35502	1mg/mL	10-15µL
Synaptophysin Primary Antibody (rabbit)	PA1-1043	120µL†	10-20µL
DyLight 650-Conjugated Goat Anti-Rabbit Secondary Antibody	84546	1mg/mL	10-15µL
High Content Analysis Buffer Kit #3	8423000	kit‡	–
Sealing Tape for 96-Well Plates, pre-cut	15036	100 sheets	1
16% Formaldehyde Solution	28906	10x1mL ampule	3mL
Poly-L-lysine coated clear-	*	–	1

bottom 96-well microplate

Note: This protocol provides general guidelines for the Synaptogenesis Assay. Further optimization of the antibodies, reagents, their preparation and the overall protocol may be required. Other reagents and dyes may be used (e.g., other DyLight Dyes), but optimization is required for each specific change.

†MA5-12826 is provided at 200µg/mL, while MA1-046 and PA1-1043 are provided at 1mg/mL for optimal functionality per application.

‡Kit components are also available separately in larger pack-sizes. Buffer Kit #3 includes Wash Buffer 10X (100mL); Permeabilization Buffer 10X (100mL); Blocking Buffer 10X (85mL); and DAPI Dye (50µL).

*This product is available through multiple suppliers.

††Prepare activator when needed. Other agonist and antagonists of the Synaptogenesis Assay can be used and should follow preparation instructions provided by the supplier.

Cell Preparation

- This protocol was performed using rat hippocampal neurons. The assay is also effective on other cell types including mouse and rat cortical neurons; however, using other cell lines may require optimization.
- Primary neuronal culture was maintained using Neurobasal™ Media, supplemented with 2mM glutamine and B27 supplement.

  Note: If primary neuronal cells are maintained using different cell culture protocols, assay optimization may be required.
- Cells were diluted to a density of 8 x 10^4 cells/mL in warmed Neurobasal Media, and 200µL of the cell suspension was added to each well of a 96-well microplate (= 16,000 cells/well for frozen rat hippocampal neuron). For frozen mouse cortical neuron, 200µL of the cell suspension was added per well from 1.6 to 3.2 x 10^5 cells/mL (recommended plating density for frozen cortical neuron is ~32,000 – 64,000 cells/well).
- Inner 60 wells were used for neuronal cell culture and outer wells were filled with media.

  Note: Handle cells gently, especially when frozen. Follow the thaw procedure provided by the manufacturer. Do not vortex cells. Minimize pipetting and handling time from thawing to cell plating.

Example Protocol for Synaptogenesis

A. Materials Required

1X Wash Buffer	Add 20mL of 10X Wash Buffer to 180mL of ultrapure water for a final volume of 200mL. Store buffer at 4°C for up to 7 days.
Formaldehyde (16%)	Immediately before use, add 1mL of the 16% formaldehyde to 15mL of 1X Wash Buffer and heat to 37°C. If using 37% formaldehyde, add 0.5mL to 18mL of 1X Wash Buffer.
1X Blocking Buffer	Add 10mL of 10X Blocking Buffer to 90mL of 1X Wash Buffer. Store buffer at 4°C for up to 7 days.
1X Permeabilization Buffer	Add 10mL of 10X Permeabilization Buffer to 90mL of ultrapure water for a final volume of 100mL. Store buffer at 4°C for up to 7 days.
Primary Antibody Solution 1	Add 24µL of PSD95 Primary Antibody and 12µL of Synaptophysin Primary Antibody to 6mL of 1X Blocking Buffer.
Primary Antibody Solution 2	Add 12µL of MAP2 Primary Antibody to 6mL of 1X Blocking Buffer.
Staining Solution 1	Add 3µL of DAPI, 12µL of the DyLight 550 Goat Anti-Mouse and 12µL of the DyLight 650 Goat Anti-Rabbit to 6mL of 1X Blocking Buffer.
Staining Solution 2	Add 12µL of the DyLight 488 Goat Anti-Mouse to 6mL of 1X Blocking Buffer.

B. Procedure – Primary Cell Culture

Note: Synapse detection is critically dependent on healthy and robust primary neuronal cells. Although MAP2 and synaptophysin markers can be detected in immature neurons, use mature neurons for PSD95 staining. Slightly different

staining results might be obtained from primary neurons depending on cell type and lot, and culture condition and time.

1. Plate 16,000 cells of hippocampal neurons in warm 200µL Neurobasal/B27 Media per well in the inner 60 wells of the 96-well plate. Add 200µL of Neurobasal Media in the outer wells of the microplate. Incubate plate at 5% CO_2.
2. Replace ~100µL of the media from each well with neurons with fresh 100µL Neurobasal/B27 Media every 3-4 days.

   Note: If the media volume in the edge well is low from evaporation, add more media to the edge well.
3. Maintain the cell culture for 4-7 weeks.

   Note: Freshly prepared primary hippocampal or cortical neurons from live tissue may be used for synapse detection earlier than 3-4 weeks of cell culture. Frozen primary cortical neurons may be used for the Synaptogenesis Assay from 21 days *in vitro* culture. The number and intensity of PSD95 puncta stain are higher in mature neuron.
4. Prepare a stock solution of the test compound in an appropriate vehicle. Dilute the test compound to appropriate concentration (i.e., 5X) in media. Add 50µL of the drug to each treatment well and add 50µL of culture medium to the control wells. Incubate the plate for a proper time at 37°C in 5% CO_2.

   Note: If the final drug concentration is critical, then skip Step 4 and follow this step – Aspirate the media from the plate and immediately add at least 100µL of the control or the drug solution (1X) to the wells. Do not expose the neuron to the atmosphere for too long or neurons will get stressed. Incubate plate for an appropriate time at 37°C in 5% CO_2.

C. Staining Procedure

1. Prepare 1X Wash, 1X Permeabilization and 1X Blocking Buffers. Make 1% Fixation Solution.
2. Set the multichannel pipette at the lowest speed. For manual pipettes, pipette as slow as possible. Pipette solutions on the wall of each well. Aspirate solutions at low speed from the corner of each well.
3. Aspirate all media and wash plate once with 100µL/well of 1X Wash Buffer.
4. Add 100µL of warmed 1% Fixation Solution to each well. Incubate plate in a fume hood at room temperature (RT) for exactly 10 minutes.
5. Aspirate Fixation Solution and wash plate twice with 100µL/well of 1X Wash Buffer.
6. Aspirate buffer, add 100µL/well of 1X Permeabilization Buffer and incubate for 10 minutes at RT.
7. Aspirate Permeabilization Buffer, add 100µL/well of 1X Blocking Buffer and incubate at RT for 15 minutes.
8. Aspirate buffer, add 100µL/well of 1X Permeabilization Buffer and incubate for 15 minutes at RT.
9. Aspirate Blocker Buffer and add 50µL/well of Primary Antibody Solution 1. Incubate plate for 1.5 hours at RT.
10. Aspirate Primary Antibody Solution 1 and wash plate twice with 100µL/well of 1X Blocking Buffer.

    Note: To avoid primary and secondary antibody cross-reaction, some antibodies are added sequentially.

11. Aspirate Blocking Buffer and add 50µL/well of Staining Solution 1. Incubate for 45 minutes at RT, protected from light.
12. Aspirate Staining Solution and wash plate twice with 100µL/well of 1X Blocking Buffer.
13. Aspirate Blocking Buffer and add 50µL/well of Primary Antibody Solution 2. Incubate plate for 1 hour at RT, protected from light.
14. Aspirate Primary Antibody Solution 2 and wash plate once with 100µL/well of 1X Blocking Buffer.
15. Aspirate buffer and add 50µL/well of Staining Solution 2. Incubate plate for 30 minutes at RT, protected from light.
16. Seal plate and evaluate on the ArrayScan HCS Reader using Neuronal Profiling BioApplication.

    Note: Scan plate on the same day of staining. Fluorescence intensity decreases with time. Because primary cell cultures are variable, appropriately adjust the parameters of the Neuronal Profiling BioApplication to detect the correct targets before scanning the plate.

Appendix A: Microscope Information

- Cells prepared and labeled according to these assay guidelines can be used and analyzed by confocal microscopy or fluorescence microscopy using the appropriate filter set(s).
- Optimization may be required when using slides, coverslips or multi-well chamber slides.
- Use image-processing software to quantify the targets. The approximate absorption/emission maxima of the fluorescent dyes are as follows:
    - DAPI = 358/461nm
    - DyLight 488 Conjugates = 494/532nm
    - DyLight 550 Conjugates = 562/572nm
    - DyLight 650 Conjugates = 646/674nm

Appendix B: Recommendation for Automation

- Plating Cells: To improve the uniformity and throughput of plating cells, use a liquid handling system such as Thermo Scientific™ Multidrop™ Combi or WellMate™ Dispensers.
- Dead Volumes: Every piece of automation instrumentation has a non-recoverable dead volume associated with it. When calculating your reagent requirements, base prime and rinse volumes on dead-volume limitations.
- Nonspecific Binding: Because of the potential of reagent interaction with large surface areas inherent to tubing, syringes and peristaltic pumps, pre-priming with reagents or pre-coating with protein blockers may be warranted.
- Mixing: Gentle mixing may be required when adding a DMSO-based solution to keep overly concentrated solutions from lying on top of the cell layer. Be careful not to dislodge cells or beads during mixing procedures.
- Cell Washing: Use an automated plate washer designed to gently wash attached cells. Be careful not to dislodge cells or beads during cell washing.

- Incubation: Minimize the time when plates with live cells are out of a controlled CO_2 environment. For best results, use an automated incubator to deliver plates to a pipetting deck.
- Exposure: Minimize operator exposure to fixative by some form of containment. Some reagents and compounds are light-sensitive; be aware of these constraints when scaling up for an automated run.
- Adapting to other plate formats: When using different plate types, adjust reagent volumes as needed.

Cited References

1. Wates, C.L., *et al*. (2005). Mechanisms of vertebrate synaptogenesis. *Annu Rev Neurosci* **28**:251-74.
2. Flavell, S.W. and Greenberg, M.E. (2008). Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. *Annu Rev Neurosci* **31**:563-90.
3. Taylor, D.L., *et al*. (2007). High content screening: A powerful approach to systems cell biology and drug discovery. *Method Mol Biol* **356**. Humana Press, Totowa, N.J.

Pierce Biotechnology
3747 N. Meridian Road
PO Box 117
Rockford, IL 61105 USA
(815) 968-0747
(815) 968-7316 fax
www.thermoscientific.com/highcontent