Chapter 2

Co-culture Synaptogenic Assay: A New Look at Fluorescence Reporters and Technological Devices

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Abstract

The mechanism underlying the differentiation of pre- and postsynaptic specifications involves the sequential and dynamic recruitment of specific molecules coordinated by bidirectional signaling across the synaptic cleft. In this chapter, we describe the co-culture assay, a useful method to evaluate cell-surface molecules through its ability to promote the recruitment of proteins required for synapse structure and function. The versatility of this simple and reliable method is illustrated by the wide variety of applications ranging from analysis of synaptogenic activity to evaluation of soluble compounds with therapeutic potential. In addition, we provide a framework to enable the co-culture assay as a tool for high-throughput studies, thereby improving the efficiency and sensitivity of this classic method in neuroscience.

Key words Co-culture assay, Adhesion molecules, Synaptogenesis, Confocal imaging, High throughput

Abbreviations

HTPS	High-throughput studies
CAMs	Cell adhesion molecules
NLG1	Neuroligin 1
EphBs	Ephrin B
LRRTM1	Leucine rich repeat transmembrane neuronal 1
SynCAMs/Necls	Synaptic adhesion molecule
HEK 293	Human embryonic kidney
COS	Cercopithecus aethiops kidney
MFD	Microfluidic devices
TIRF	Total internal reflection fluorescence
CFP	Cyan fluorescent protein
P0	Postnatal day 0
Fiji	ImageJ image processing package (fiji.sc/Fiji)

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Amaxa	Electroporation system for gene delivering (bio.lonza.com)
MATLAB	Matrix laboratory technical computing language (mathworks.com)
GNU Octave	Matrix laboratory technical computing language (gnu.org/software/octave/)
Imaris	Interactive microscopy image analysis software (bitplane.com)

1 Introduction

Development and maintenance of synaptic architecture is considered one of the most fundamental processes underpinning neuronal health and functionality. One technique that has become fundamental for the study of synapse-organizing factors is the co-culture assay, a classic method in neuroscience with a pivotal role in the identification and characterization of transmembrane adhesion complexes as major players in synapse development [1–3]. The main outcome of this experimental approach is to provide a quantitative measurement of synaptogenic activity, based on the extent to which neuronal proteins are recruited to putative hemi-synapses as a result of the cell-surface protein expression on non-neuronal cells [4, 5]. Therefore, this reliable and convenient technique provides a comparative method not only for the analysis of several trans-synaptic molecules in parallel, but also for exploring cooperative actions between them.

The substantial contribution of the co-culture assay revealing the synaptogenic activity of diverse families of neuronal cell adhesion molecules (CAMs), such as Neuroligins and Neurexins [3, 6], EphBs and ephrinBs [7, 8], LRRTMs [9], SynCAMs/Necls [2], and netrin G ligands (NGLs) [10], has been crucial to further our understanding of mechanisms that regulate synapse formation and maintenance. Thus, studies in vivo using knockout mouse models for EphBs/ephrinBs [7, 8, 11, 12] and neurexins/neuroligins [13, 14] have established these trans-membrane proteins are more relevant for maintaining the integrity of synaptic junctions in vivo than they are essential for initiating the synapse formation process. Therefore, complementary studies on whole animal systems offer the opportunity to investigate these proteins in a physiological context enlightening more precise descriptions of their functions during synapse assembly, maturation, and stability rather than focusing only on synaptogenic activity [15].

The co-culture assay's versatility is outlined along this chapter by the detailed description of useful modifications together with the discussion of recent technical tools that improve its precision and sensitivity and also provide enhanced reliability in its adaptation for high-throughput studies (HTPS). Thanks to a new set of technological devices this assay can be upgraded to conduct HTPS with the aim of performing large screens for novel soluble factors, molecules, and drugs with biological and/or biochemical activities that regulate synapse assembly [16–19]. In this context, screens

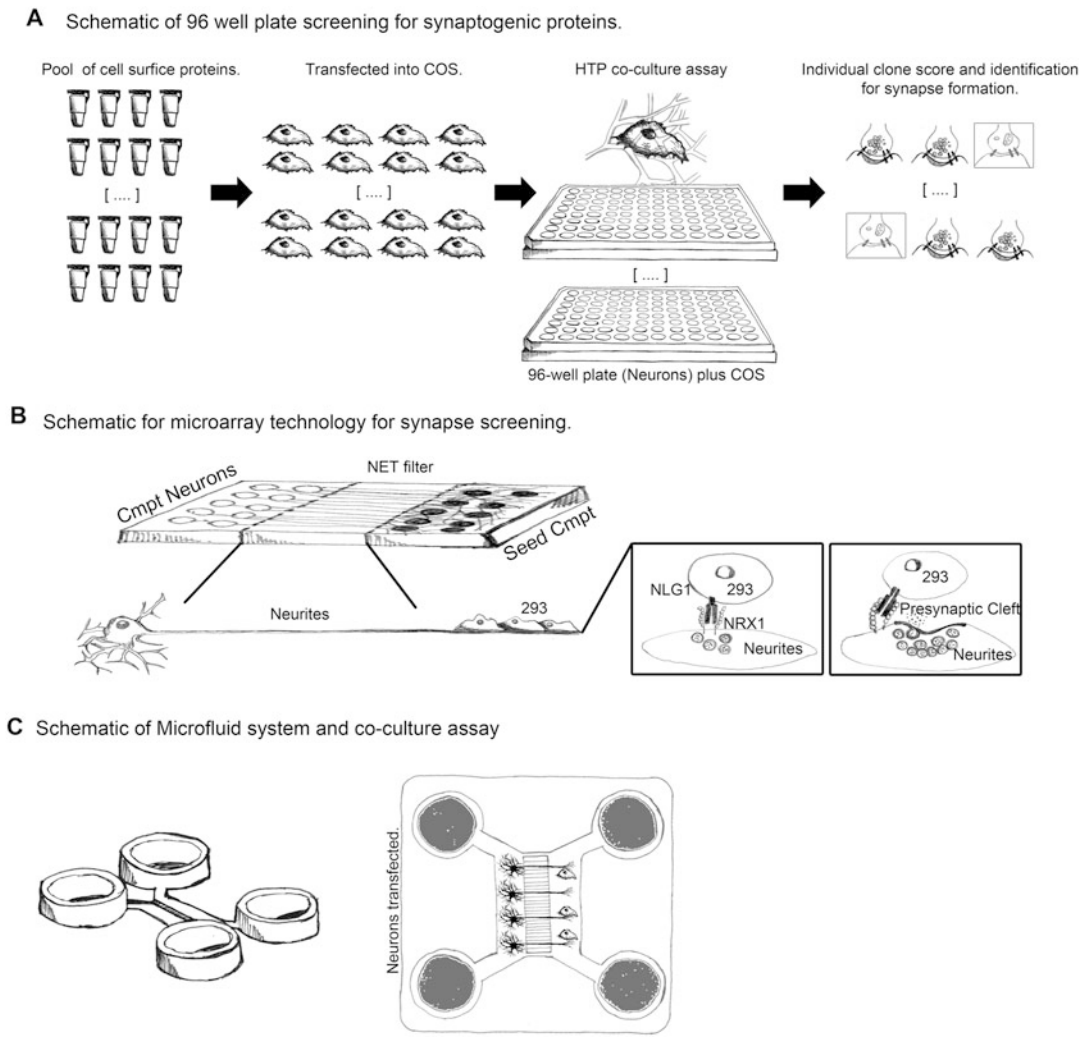

Fig. 1 Devices for large-scale co-culture assays. (**a**) Synapse Microarray Technology. The schematic representation shows the chambers where neurons are loaded (neuron cmpt) and the chamber where the co-culture is placed (seed cmpt). Heterologous expression of Neuroligin 1 (NLG1) in HEK293 will induce presynaptic differentiation in neurons by recruitment of presynaptic proteins in the seed compartment. (**b**) Large screening of synaptogenesis-inducing molecules using a 96-well format and imagining with an HTP screening microscope. Several cell-surface proteins are transfected in HEK293 and seeded as multiple replicates over neurons previously plated in each of the wells. (**c**) Microfluidic culture devices that isolate the neuronal processes from the cell bodies via a series of interconnected axon-guiding microchannels

can be performed using an HTPS microscope in 96-well plate formats to allow large scale imaging and analysis of synaptogenic activity (Fig. 1a). Alternatively, synapse microarray technology enables neurites to extend through microchannels and form dense networks in compartments distinct from those where their cell bodies reside [17]. Since this platform has a controlled array of microwells (Fig. 1b), the induction of presynaptic structures will be selectively established in predetermined positions and therefore

will restrict imaging space, decreasing search time for synaptogenic events, and increase the reproducibility of co-culture assays. Other interesting platforms are microfluidic devices (MFD), originally described for the study of axon regeneration [19], and subsequently used as co-culture chambers [16, 18]. A typical MFD has two compartments, the soma and the axon compartment, connected through arrays of axon-guiding microchannels (Fig. 1c). This platform also enables multiple parallel treatments and/or co-cultures, with fluidic isolation as key feature, since axons and non-neuronal cells are placed in different compartments from the neuronal somata.

In parallel to the technological devices described above, there are several new molecular biological tools that are particularly useful as well. New fluorescent probes with improved sensitivity and temporal resolution for studding synaptic activity are available and used intensively in the field. Thus, co-culture assays can be used to assess the induction of new synapses, not only through endogenous synaptic protein localization (Table 1), but also activation of

Table 1
Antibodies for synaptic markers used in co-culture assays

Description	Target antigen	Provider
Axons	Tau GAP-43	Pierce [30] Pierce [32]
Dendrites	MAP2	Chemicon [33]
GABAergic presynaptic sites	GAD65	Chemicon [15]
GABAergic postsynaptic sites	Gephyrin GABAA receptor subunit γ 2	Synaptic system [34] Alomone [33]
Glutamatergic postsynaptic sites	PSD-95 NMDA receptor subunit NR1 Glutamate receptor subunit GluR1	Neuromab [35] Synaptic systems [15] Upstate [26, 33]
Presynaptic actives zones	Bassoon	ENZO [36]
Synaptic vesicles	Synapsin 1 Syntaxin 1 Synaptophysin 1 Synaptotagmin 1 SV2	Chemicon [23] Synaptic systems [37] Synaptic systems [23, 37] Synaptic systems [38] Developmental studies Hybridoma bank [39]
GABAergic synaptic vesicles	vGAT	Synaptic systems [40]
Glutamatergic synaptic vesicles	vGlut1	Neuromab [35]

new synaptic terminals. This has already been done with synaptic proteins tagged with pHluorin and GCaMP2, a pH-sensitive GFP and a genetically encoded calcium sensor, respectively [20–24]. In addition, many new biosensors show great potential for this assay: GCaMP6 [25] an updated calcium sensor for imaging neural activity, iGluSnFR [26] to visualize glutamate dependent synaptic transmission, and ArcLight-S249 to detect single action excitatory potentials in individual neurons and dendrites [27].

The following protocol will describe the complete panorama of this method with detailed descriptions of the classic co-culture assay from the basics of neuronal culture preparation to elements of analysis using the most common software available, including the new technological devices that may be used for HTPS.

2 Materials

2.1 Reagents

1. Heterologous Cells: HEK 293 or similar (*see* **Note 1**).
2. DMEM: Dulbecco's Modified Eagle's Medium.
3. CMF-HBSS Solution: Hanks' Balanced Salt Solution (HBSS), 1 mM Sodium Pyruvate, 50 U/mL Penicillin/Streptomicyn, 10 mM HEPES, filtered through 0.2 μm under sterile conditions.
4. Papain Solution: 25 U/mL Papain, 20 U/mL DNAse, 1.5 mM CaCl2, 0.75 mM EDTA, 0.75 mM NaOH, l-Cysteine 5.5 mM in HBSS, filtered through 0.2 μm under sterile conditions.
5. Trypsin/EDTA: 0.25% Trypsin, 0.5 mM EDTA in HBSS pH = 7.0–7.6.
6. Plating Medium: Minimum Essential Medium (MEM), 10% Fetal Bovine Serum (FBS), 1× Glutamax (e.g., Life technologies), 5 mM HEPES, 50 U/mL Penicillin/Streptomycin, 0.6% glucose, filtered through 0.2 μm under sterile conditions.
7. Neurobasal Medium: Neurobasal (Life Technologies), 1× B27 supplement (Life Technologies), 1× Glutamax, 50 U/mL Penicillin/Streptomicyn, filtered through 0.2 μm under sterile conditions.
8. HEK293 Medium: DMEM, 10% FBS, 1.2 mM Sodium Pyruvate, 50 U/mL Penicillin /Streptomycin, filtered through 0.2 μm under sterile conditions.
9. Neurobasal-ARAC Medium: 2 μM Cytosine-β-d-arabinofuranoside in Neurobasal Medium.
10. Matrigel Solution: CMF-HBSS Solution and Matrigel® (as per lot dilution instructions, BD).
11. Transfection reagent: e.g., FuGENE 6 Transfection Reagent (Promega) or Lipofectamine LTX (Life Technologies).

12. PFA Solution: 4% Paraformaldehyde, 4%Sucrose in PBS, pH to 7.4 and filter, store at 4 °C for no more than 2 weeks
13. Permeabilization Solution: 0.1% Triton X-100 in PBS.
14. Blocking Solution: 3% FBS in PBS.
15. Mounting medium with anti-fade: e.g., Aqua-Mount (Thermo Scientific).

2.2 Instrumentation

1. Dissecting tools (sterilized): fine-tipped forceps (for example, Dumont no. 5), microdissecting scissors (Vannas-style spring scissors).
2. Tissue culture incubator at 35.5 °C with humidified, 5% CO_2 atmosphere.
3. Sterile plastic ware: 100 × 20 mm tissue culture dishes, 75-cm^2 tissue culture flasks, 24- and 6-well tissue culture plate.
4. Sterilization filter units: syninge-driven, and cups of 250 and 500 mL.

3 Methods

3.1 Hippocampal Cultures

1. Prepare coverslips in 24-well plates by adding 150 μl Matrigel Solution per coverslip and leave for at least 2 h in the incubator. Aspirate the Matrigel Solution leaving a thin layer coating the glass. Dry the coverslips for a few minutes. Wash the coverslips two times with plating medium and finally add 1 mL plating medium. Store the 24-well plate in the incubator until hippocampal neurons are ready to be seeded.
2. Dissect out the brain from one P0 rat pup using an approved method of euthanasia (*see* Fig. 2a); immediately store the brain in CMF-HBSS Solution on ice until you have collected all needed samples. One brain will provide enough cells for 12 coverslips of neuron-glia culture.
3. In the laminar flow hood, collect all brains and place them in a dish with CMF-HBSS Solution on ice. The brains should be completely submerged until the hippocampi are removed from all the samples.
4. Carefully remove the meninges and dissect out the hippocampi with fine forceps under the dissection microscope. Collect the hippocampi in a 15 mL tube with CMF-HBSS Solution on ice (*see* Fig. 2a).
5. In a tissue culture hood, wash the hippocampi with cold CMF-HBSS Solution two times.
6. Aspirate the liquid and add Papain Solution to the hippocampi. Incubate them for 30 min at 37 °C.

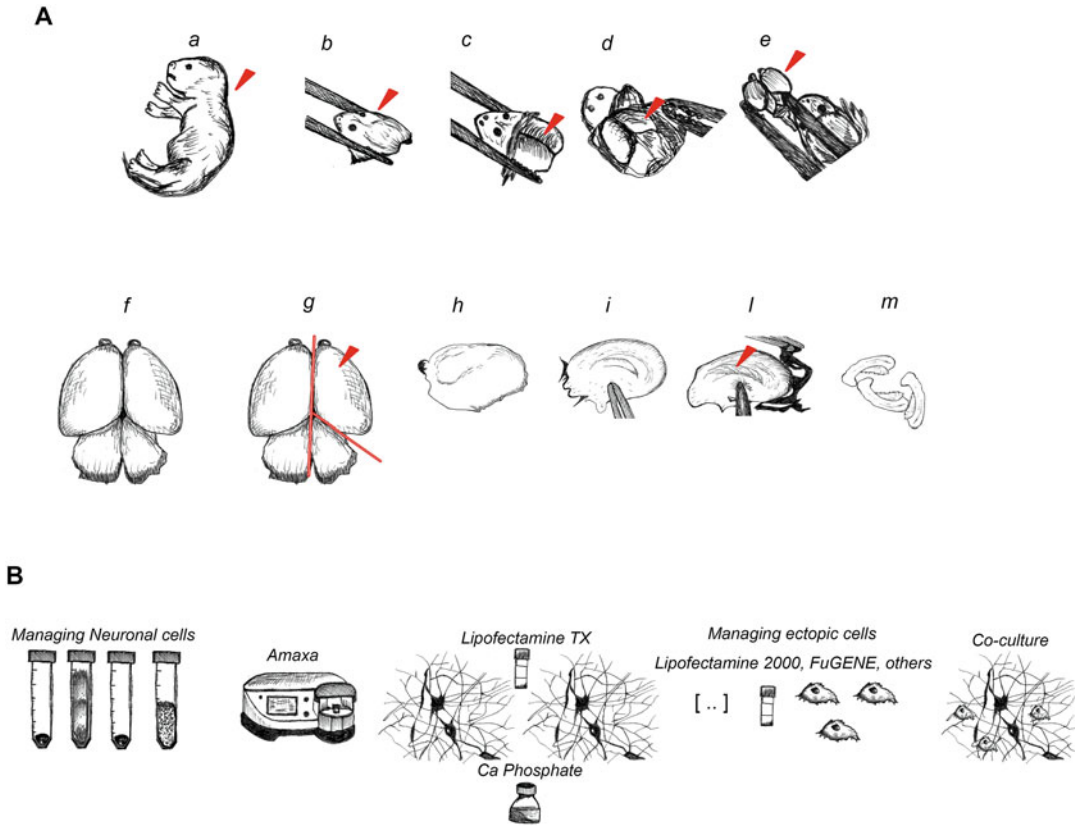

Fig. 2 Schematic representation of hippocampus dissection and cell transfection. (**a**) Flow chart showing dissection of hippocampus from a P0 rat brain. Take out the brain (*steps a, b, c, d,* and *e*) and once the meninges have been removed (*f*), separate the cortical rind from the thalamo-cortical fiber tract (step *g*). Finally, the hippocampi can be removed using forceps (*steps h*, *i*, *l*, and *m*). (**b**) Overexpression of synaptic proteins in neurons can be performed using different protocols. Neurons can be transfected according to the Amaxa protocol [31] before plating, or neurons can be transfected using Lipofectamine LTX or Calcium Phosphate at 5–7 DIV. HEC293 cells can be transfected with the commercial regent FuGENE as is described in Subheading 3.2

7. Aspirate the papain solution and wash the hippocampi twice with Plating Medium leaving them finally in 2 mL of medium.
8. Triturate the hippocampi gently by passing the suspension 10–12 times through a glass Pasteur pipette previously fire-polished (hold glass pipette for 20–30 s close to a flame until the edges become smooth (*see* **Note 1**)).
9. Allow un-dissociated pieces of hippocampus to settle by gravity for 2–3 min.
10. Determine cell density using a hemocytometer. The yield should be around 500,000 cells per hippocampus. Plate the dissociated neurons at 30,000–40,000 cells per well (150 μL approx.) on the plate that was prepared in **step 1**. Place the neurons in the cell culture incubator for 20–24 h.

11. Aspirate the Plating Medium and add 1 mL of Neurobasal Medium per well. No washes are required in between the medium change.
12. Neurons at 6–8 Day In Vitro (DIV) are recommended for co-culture. Alternatively, neurons can be transfected for expression of synaptic markers tagged with fluorescent probes (*see* **Note 2** and Fig. 2b).

3.2 HEK293 Cell Culture

1. HEK293 cells are plated with a seeding confluence of 15,000 cell/cm^2. The working volume for one well with a 9.4 cm^2 surface area is 1 mL (*see* **Note 3**). This must be done when neuronal cultures reach 4–6 DIV.
2. 24 h post-seeding, HEK293 cells are transfected using the vector of interest, tagged with a fluorescent protein, or co-expressing soluble fluorescent protein as a marker of transfected cells. Successful transfections require plasmid DNA with a 260/280 nm absorption ratio close to 1.8 in addition to previous optimization of Transfection-reagent:DNA ratio (*see* **Note 4**).
3. For transfection, we will describe the procedure for standard lipofection using the FuGENE transfection reagent. To prepare transfection solutions, first mix FuGENE with pre-warmed DMEM (serum-free, no antibiotics) to a final volume of 50 μL per well of cells that will be transfected (*see* **Note 5**). Vortex and incubate for 5 min.
4. Prepare an additional tube with a mix of plasmid DNA and DMEM to a final volume of 50 μL per each well to be transfected.
5. Add the 50 μL of DNA solution to the 50 μL of diluted FuGENE and incubate at least for 15 min at room temperature (*see* **Note 6**).
6. Add 100 μL of the Fugene:DNA complex solution over each well of cells in a drop-wise manner. To ensure a homogenous distribution along the surface, mix solution with the medium by gentle rotational movements.
7. Return the cells to the incubator for 24–48 h. Check the transfection efficiency on an epifluorescence microscope to visualize fluorescence-expressing cells. Usually, 48 h post-transfection HEK293 cells show high levels of expression.

3.3 Neuron-HEK293 Cell Co-culture

1. Harvest HEK293 cells 24–48 h post-transfection, by adding 500 μL Trypsin/EDTA per well.
2. After a few minutes place the suspension in 4.5 mL of HEK293 Medium and pellet the cells by centrifugation at 500 × *g* for 5 min.
3. Resuspend the cells in 2 mL Neurobasal Medium.

4. Determine the cell density using a hemocytometer.
5. Dilute the cells in order to have 20,000cells/100 µl and add ARAC (22 µM) to avoid over growth of HEK293 cells after co-culture with neurons (*see* **Note 7**).
6. Seed 20,000 HEK293 cells (100 µl) per well of neurons at 6–8 DIV. Maintain the co-culture in the incubator for 24–48 h (*see* **Note 8**).

3.4 Co-culture Immunostaining

1. Wash the co-culture with PBS at RT, then fix the coverslips by bathing in PFA Solution for 15 min at RT. This step is critical since longer fixations can affect immunofluorescence quality (*see* **Note 9**).
2. Wash the cells carefully three times using PBS.
3. Incubate with Permeabilization Solution for 15 min at. Wash cells three times with PBS.
4. Add Blocking Solution for 1 h at RT.
5. Incubate with primary antibody for synaptic markers diluted appropriately in Blocking Solution for 1 h at RT (*see* **Note 10**). Then wash three times with PBS. Representative images of presynaptic proteins vGluT1 and Bassoon are shown in Fig. 3.
6. Add the secondary antibody diluted appropriately in Blocking Solution and incubate for 1 h at RT. Wash three times after remove the secondary antibodies.
7. Mount the coverslips using a drop of mounting medium with optional anti-fade (i.e., Aqua-Poly/Mount solidifies after 24–48 h/20–50% humidity). The coverslip can be stored in the dark at 4 °C.

3.5 Image Acquisition

The settings for image acquisition depend strongly on the fluorophores selected for the assay and the experimental conditions. Spinning disk or TIRF microscopy is suggested when the hemisynapse must be visualized using live imaging of fluorescent reporters such as pH-sensitive targeted synaptic proteins. The opportunity to combine different biosensors, specific synaptic proteins, and microfluidic device makes the possibilities of the co-culture assay compatible with a large spectrum of applications in neuroscience.

The "classic" co-culture assay experiment using the protocol described in this chapter can be easily analyzed using a confocal microscope with the same image acquisition settings for each sample of an experiment (*see* Fig. 3). In this case, quantification of presynaptic protein recruitment is performed using Z-section images that were converted to maximal projection images (*see* Fig. 3a). Transfected HEK293 cells, with control or Neuroligin 1 overexpression constructs, in close proximity to neurons were selected based on CFP labeling with the aim of measuring the fluo-

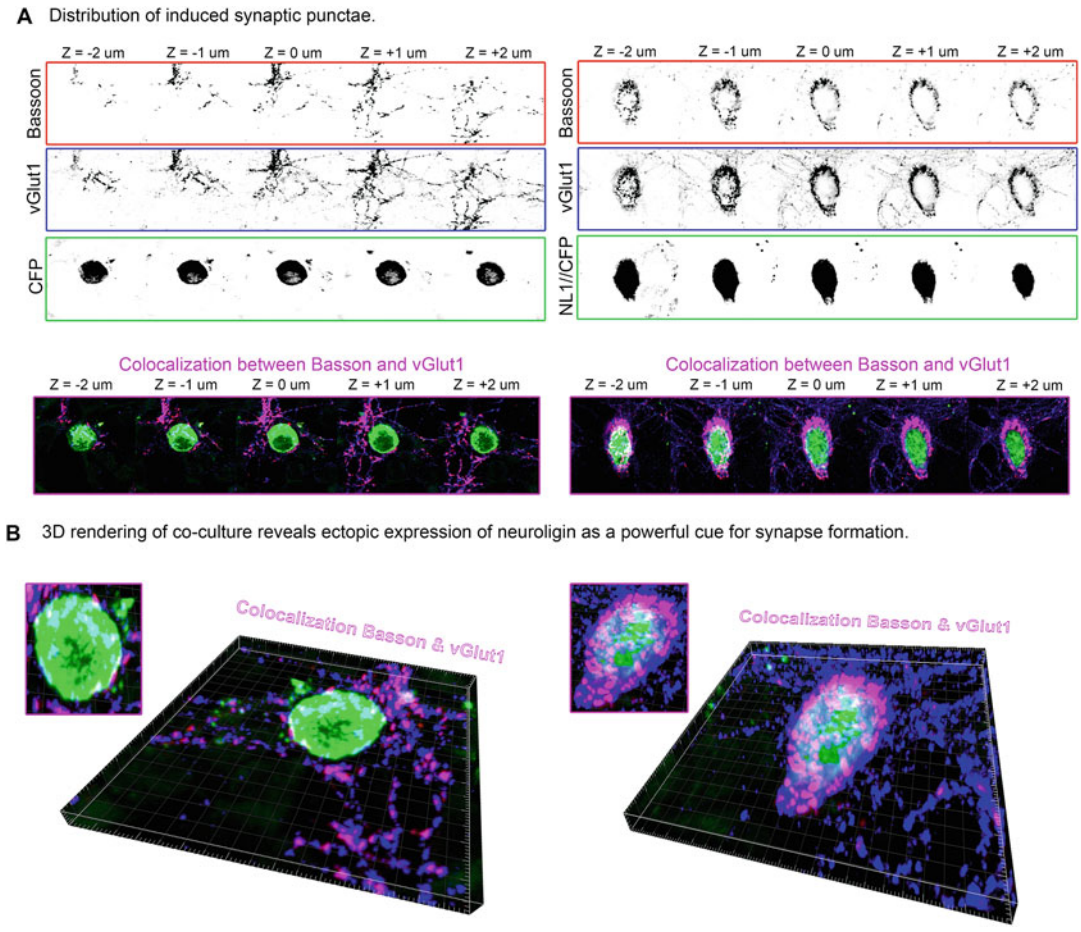

Fig. 3 Co-culture assay image acquisition. (**a**) Visualization of unmerged serial optical sections for presynaptic proteins Bassoon (*red*) and vGlut1 (*blue*) after 48 h of co-culture with HEK293 cells expressing Neuroligin 1 with CFP (CFP/NLG1) and CFP alone as negative control (**a**). *Bottom panel* shows the colocalization of vGlut1 and Bassoon with CFP (*green*) that is color-coded in *violet*; only the expression of NLG1 induces the recruitment of synaptic markers. (**b**) 3D rendering of neuron-HEK293 co-culture showing synaptic proteins that co-localize with heterologous expression of NLG1 (**b**). Presynaptic vGlut1 and Bassoon clusters are distributed along HEK cell surfaces that express NLG1 (*violet*)

rescence intensity for both red (Bassoon) and far-red channels (vGlut1) in the region of interest.

3.6 Analysis

In this chapter, we give a practical example of analysis of co-culture data obtained from a point-scan confocal microscope (*see* Fig. 3). Immunostaining of mixed co-cultures for the presynaptic markers vGlut1 and Bassoon was performed at 10 DIV, and images were acquired by confocal microscopy with the modalities described above. As control, HEK293 expressing the fluorophore probe alone (i.e., CFP) should be analyzed in parallel with HEK293

expressing the candidate protein tagged with a fluorescent probe, i.e., the bicistronic vector encoding both CFP and the postsynaptic adhesion molecule Neuroligin 1 (*see* Fig. 3).

A variety of standard software has been intensively used for co-culture quantifications allowing the automatization of the process, among the most popular are Matlab script (*see* **Note 11**), GNU octave and ImageJ software [28] (*see* **Note 12**). The quantification of the HEK293 cell surface area fraction immune-positive for synaptic markers requires the initial establishment of the proper threshold for the channel with the fluorescence signal from a given HEK293 cell (in this case CFP). This will help to define the perimeter of the cell, which subsequently will be used as a mask for the second and third channels that correspond to the synaptic markers. The readout most commonly used, as we mentioned above, is the fraction area positive for synaptic marker over the total surface area (perimeter of the HEK293 cell). To obtain this value the user should divide the surface area of either channel two or three by the surface area of channel one (CFP) which was previously obtained by applying the threshold and mask steps using the imaging software. Critical for accurate quantitative analysis is using identical acquisition settings for every image in addition to performing the image analysis with constant thresholds. Representative images of the synaptic marker distribution on the ectopic Neuroligin-expressing cell surface after the z-stack montages were loaded (*see* Fig. 3a) and converted to maximal-projection images using imaging software (*see* Fig. 3b, upper left panels).

Optionally, representative images of 3D rendering can be achieved using Imaris software (*see* **Note 13**). One of its main advantages is the relatively easier way to produce 3D rendering compared with other software. In addition, Imaris version 7.4 and newer can access real-time data from Fiji or ImageJ (*see* **Note 12**), making the 3D rendering operation more efficient and less time-consuming (*see* Fig. 3b). The images must be in tiff format to be processed by the software and initially the image sequence should be loaded in Fiji using the RGB matrix to see the different channels. Open the Imaris software and select Fiji task from the main menu, select the image to analyze from Fiji using the menu window. Once the software automatically loads the 3-channel z-stack image from Fiji, the 3D rendering will be immediately build. Although Imaris software is able to import other image formats, the import process itself can lose spatial information. One way to overcome this issue is doing the redefinition of spatial parameters with very simple and intuitive operations.

3.7 High-Throughput Devices

1. HTP screening microscope.

The assay is an extension of the co-culture methodology and analysis described above. The great advantage is that it is possible to use 96- or 384-well plates to screen a large pool of candidates of

synaptic inductors simultaneously. The possibility of using different cell types for co-culturing and alternative libraries of chemical compounds, and the possibility of multiple repetitions make the use of an HTP device incredibly useful for the co-culture assay with high statistical power. Expression library pools are now available from many vendors and the cost has become relatively affordable.

The challenges of this approach are related to the large scale transfection cost, antibody and related reagents, but mostly to the hardware, since a microscope with HTP capability may be a significant expensive. There are two main options for microscopes with HTP capabilities: Epifluorescent system (e.g., ImageXpress, Molecular Device) or spinning disk system (e.g., Opera /Operetta, Perkin Elmer). Already some laboratories have used screening approaches successfully, identifying LRRTM1 as a synaptogenic factor [9]. These commercial microscopes are accompanied by strong customer service, with in-house training and the possibility of integrating the screening software to automatize your co-culture assay to your experimental needs.

A useful alternative for a robust and high-content method is culturing neurons on micro-patterned substrates comprising arrays of individual micron-scale dots coated with proteins that trigger neuronal development [29]. The main advantage of this alternative method is that it can be used with traditional microscopy techniques in addition to be useful for the screening of pharmaceutical compounds modulating synaptogenesis.

2. Microfluidic devices.

These devices can be used to enrich the imaging area in pre- or postsynaptic terminals, providing a standard and dense region to assay synapse formation in the co-culture assay. Briefly described, etched soft substrates are attached to the imaging device (slide or chamber), with small wells that will be used to plate neurons/heterologous cells. In addition, fine-etched channels (approx. 250–100 μm) that allow the extension of neuronal processes, but not neuronal cell bodies, mediate the communication between wells. Therefore, we can plate neurons in one or several wells and subsequently these neurons will assemble synapses on heterologous cells located in adjacent wells. Neurons can be prepared as described in Subheading 3.1. The density of the neuronal culture must be around 50,0000 cells/mL, but this device has been used mainly with neuronal cultures from E18 embryos [30]. The platform to implement this assay can be designed manually and fabricated in PDMS using soft lithography and replica molding [18, 19], but is also commercially available (e.g., Millipore, AX45005 and XONA Microfluidics). Neurons and HEK293 cells are seeded in different compartments as is shown in Fig. 1c. For the synaptic markers, immunostaining follows the protocol described in Subheading 3.4, though an additional step may be necessary to block nonspecific binding using 5–10% goat serum or BSA.

4 Notes

1. Dissociation of hippocampi is a critical step; to avoid low viability be very gentle during the pipetting step and do not use pipettes with a very narrow diameter.
2. To analyze synaptic activity-inducing factors, neurons can be transfected with synaptic markers tagged with fluorescent probes as pHlurorin or GCamP (*see* Fig. 2b). For this assay, neurons are transfected via nucleofection (Amaxa) before plating (*see* Subheading 3.1, **step 9**). Alternatively, neurons can be transfected at 7–9 DIV accordingly with published methods with Lipofectamine LTX [15] or Calcium Phosphate [31].
3. Other heterologous cells also can be utilized successfully in this assay. PC12 cells endogenously express several neuronal proteins, which is advantageous if neuron-specific posttranslational modification is required. However, the neuronal proteins expressed by PC12 cells can interfere with the interpretation of results. COS7 cells are also a viable alternative to HEK293 cells as they have greater areas with flatter surfaces.
4. Previous optimization of FuGENE:DNA ratio can be made using the provider guidelines. In particular for HEK93 cells, 3 μL of FuGENE and 1 μl DNA (3:1 ratio) give high transfection efficiency.
5. To avoid adverse effects on transfection efficiency do not allow FuGENE to come into contact with the walls of the tube containing DMEM. It is recommended to make a master mix of FuGENE solution depending on the total number of wells to be transfected. Each well of transfected HEK293 cells in this protocol will provide enough cells for more than four co-culture coverslips.
6. Incubation can continue up to 45 min without affecting the transfection efficiency, although there is some variation depending on the cell line used for the co-culture assay. For HEK293 cells, incubations up to 20 min are enough to get high transfection efficiency at 24–48 h post-transfection.
7. Since high ARAC concentrations can be toxics for neurons, ARAC should be diluted at 2 μM final concentration with Neurobasal Medium.
8. Volumes up to 100 μL of HEK293 cells per 1000 μL well of neurons are optimal for co-culture conditions.
9. Preparation of paraformaldehyde (PFA) solution is a critical step. Always adjust the pH close to 7.4 and filter the solution. Preferably use fresh aliquots of PFA for each experiment, as it is only stable for 2 weeks at 4 °C. Alternatively aliquots can be kept at −20 °C and discarded after use to avoid freeze- thaw cycles.
10. The spatial features and intensity of synaptic puncta revealed by each synaptic marker will directly impact the quality of the

quantification process. Although the staining conditions should be established empirically for each primary antibody, improvements can be made by overnight incubation at 4 °C. A detailed description of primary antibodies useful for co-culture quantifications is shown in Table 1.

11. MathWorks MatLab® (http://www.mathworks.com) or Gnu Octave (http://www.gnu.org/software/octave/) is high-level interpreted language, primarily intended for numerical computations with extensive graphics capabilities for data visualization and manipulation. The Octave language is quite similar to Matlab so that most programs are easily portable. As Matlab is a commercial package, it is richer in functions and imaging analysis capabilities. For both packages an intermediate knowledge of computer programming is required.
12. ImageJ (http://imagej.nih.gov/ij/download.html) is straightforward software for image analysis. Several versions have been developed, and are distributed via different institutions: Fiji, MCMB, MIC, etc. Currently, ImageJ FIJI is certainly the most versatile and open software. If the user has a good knowledge of Java, they can easily build and/or modify the plugins according to their own needs.
13. Bitplane Imaris® (http://www.bitplane.com/imaris) is professional software for bio-imaging analysis with modules for multidimensional rendering, co-visualization, and quantification of particle tracking and morphology. The software can be integrated with Matlab, so the user can build their own specific micro-scripts using MatLab extensions.

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