TECHNICAL COMMUNICATION

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Cell-based immunofluorescence assay for screening the neurogenesis potential of new drugs in adult hippocampal neural progenitor cells

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Preclinical studies have suggested that increased adult neurogenesis in the hippocampus might have potential therapeutic effects for Alzheimer's disease and depression; therefore, it is a target for the treatment of some brain diseases. In this technical communication, we propose a cell-based fluorescence assay to study the neurogenesis of adult hippocampal progenitor cells that can be used for high-throughput screening of drugs promoting neurogenesis. Three fluorescent dyes (DAPI, Alexa Fluor 488, and Alexa Fluor 594) and a fluorescence spectrophotometry reader were used, which confirmed that the mutual interference of the three fluorescent dyes is very low. We used this cell-based fluorescence assay to evaluate the effects of three neurotrophic factors, ciliary neurotrophic factor (CNTF), insulin-like growth factor 1 (IGF-1), and IGF-2 on the promotion of neurogenesis in adult hippocampal neural progenitor cells. The fluorescence intensity ratio of the neuronal marker, class III β-tubulin, to the housekeeping protein, glyceraldehyde 3-phosphate dehydrogenase, or nuclear staining dye, DAPI, in CNTF-treated cells was significantly higher than in control cells. The ratios in IGF-1- and IGF-2-treated cells were slightly higher under higher cell density conditions. These results are consistent with those in previous reports; therefore, this report proved the efficacy of this method. Taken together, the results showed that this simple, rapid, and economical cell-based immunofluorescence assay could be a powerful tool for the rapid screening of drugs that promote adult neurogenesis.

Key words: cell-based assay, fluorescent labeling, adult neurogenesis

Substantial neurogenesis and continuous supplementation of newborn neurons in the hippocampus and striatum of human adults indicate that neurogenesis may have important implications for brain function (Bergmann et al., 2015; Boldrini et al., 2018). Adult neurogenesis in the hippocampus is important for memory coding and mood regulation. Furthermore, alterations in adult neurogenesis are associated with several brain diseases, such as neurodegenerative diseases, ischemic stroke, and psychiatric disorders. Some symptoms of these diseases can be explained by the dysregulation of adult neurogenesis (Winner and Winkler, 2015; Toda et al., 2019). Preclinical studies have suggested that increased adult neurogenesis in the hippocampus has potential therapeutic benefits in patients with Alzheimer's disease and depression (Iqbal and Grundke-Iqbal, 2011; Miller and Hen, 2015; Han et al., 2016). This has led to adult neurogenesis as a target for the treatment of some brain diseases.

Several methods can be used to detect neurogenesis, including *in vivo* and *in vitro* labeling with the thymidine analogue, bromodeoxyuridine (BrdU), endog-

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enous cell-cycle markers, and cell stage and lineage commitment markers (Kuhn et al., 2016). Each method has its own strengths and limitations; however, when several compounds require quick screening to determine whether they promote neurogenesis, these methods may be unsuitable as they require several reagents and are time-consuming. Therefore, we propose a cell-based fluorescence assay to study neurogenesis in adult hippocampal progenitor cells. This method is advantageous because it uses 96-well fluorescence plate readers, which enables a large amount of experimental data, and dozens of compounds, to be read simultaneously. This method could be effective in the screening stages of drug development. In addition, this method is more economical than those previously described.

The fluorescent dyes used for the cell-based fluorescence assay were DAPI (blue), Alexa Fluor 488 (green), and Alexa Fluor 594 (red). The fluorescence spectrophotometry reader was the Cyto Fluor Multi-Well Plate Reader, series 4000 (PerSeptive Biosystems, MA, USA) with standard filter sets of the instrument used. The center wavelength/full bandwidth of excitation filter and emission filter for DAPI are 360/40 and 460/40; for Alexa Fluor 488 are 485/20 and 530/25; for Alexa Fluor 594 are 590/20 and 645/40, respectively. The online tool, Fluorescence Spectra Viewer (https: //www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html), showed that there was almost no mutual interference under these conditions.

To further confirm this result experimentally, we compared the fluorescence intensity (FI) values of single fluorescent dyes and the combination of the three fluorescent dyes. The fluorescent dyes were diluted in 0.05 M Tris-buffered saline (TBS, 50 mM Tris-Cl, 150 mM NaCl, pH 7.6) at three dilutions (1, 1/3, and 1/9). The concentrations of DAPI (Thermo Fisher Scientific, Waltham, MA, USA) were 50, 16.7, and 5.6 μg/ml. The concentrations of Alexa Fluor 488-conjugated goat anti-rabbit (Alexa488) or Alexa Fluor 594-conjugated goat anti-mice (Alexa594) antibodies (Thermo Fisher Scientific) were 2.0, 0.67, and 0.22 μg/ml. In total, 100 μl of each sample was added into the wells of a 96-well fluorescence-based assay microplate (Thermo Fisher Scientific). Three wells were used per sample. The plate was read on a fluorescent spectrophotometer. The results showed that for a single fluorescent dye sample, the FI value is very low if read at a non-corresponding filter setting and the differences in FI value in the mixture of fluorescent dyes compared to a single fluorescent dye were less than 7%. Therefore, the experimental results are consistent with the analysis results from the Fluorescence Spectra Viewer; that is, the mutual interference of the three fluorescent dyes is very low (Tables I, II, and III).

We used this cell-based fluorescence assay to evaluate the effects of three neurotrophic factors on promoting neurogenesis of adult hippocampal neural progenitor cells; ciliary neurotrophic factor (CNTF) significantly induced neuronal differentiation, while

Table I. The OD value of single and mixed fluorescent dyes at filter set 360 nm/450 nm, gain at 30.

Fluorescent dyes	OD value			Mean	The changes (%) compared to a single fluorescent dye	Mean
DAPI (50 μg/ml)	1063	1057	1078	1066.0		
DAPI (16.7 μg/ml)	489	499	501	496.3		
DAPI (5.6 μg/ml)	179	184	182	181.7		
Alexa488 (2.0 μg/μl)	1	1	1	1.0		
Alexa488 (0.67 μg/μl)	1	1	1	1.0		
Alexa488 (0.22 μg/μl)	1	1	1	1.0		
Alexa594 (2.0 μg/μl)	1	1	1	1.0		
Alexa594 (0.67 μg/μl)	1	1	1	1.0		
Alexa594 (0.22 μg/μl)	1	1	1	1.0		
DAPI (50 μg/ml), Alexa488 (2.0 μg/ml), Alexa594 (2.0 μg/ml)	1011	1009	999	1006.3	–5.6	–2.1
DAPI (16.7 g/ml), Alexa488 (0.67 μg/ml), Alexa594 (0.22 μg/ml)	485	487	482	484.7	–2.4	
DAPI (5.6 μg/ml), Alexa488 (0.22 μg/ml), Alexa594 (0.22 μg/ml)	183	183	188	184.7	1.7	

Table II. The OD value of single and mixed fluorescent dyes at filter set 485 nm/530 nm, gain at 50.

Fluorescent dyes	OD value			Mean	The changes (%) compared to a single fluorescent dye	Mean
DAPI (50 μg/ml)	2	1	2	2.0		
DAPI (16.7 μg/ml)	2	1	1	1.0		
DAPI (5.6 μg/ml)	0	2	1	1.0		
Alexa488 (2.0 μg/μl)	702	710	679	697.0		
Alexa488 (0.67 μg/μl)	238	241	233	237.0		
Alexa488 (0.22 μg/μl)	80	83	77	80.0		
Alexa594 (2.0 μg/μl)	0	0	0	0.0		
Alexa594 (0.67 μg/μl)	0	0	0	0.0		
Alexa594 (0.22 μg/μl)	0	1	2	1.0		
DAPI (50 μg/ml), Alexa488 (2.0 μg/ml), Alexa594 (2.0 μg/ml)	710	704	696	703.0	0.9	2.4
DAPI (16.7 g/ml), Alexa488 (0.67 μg/ml), Alexa594 (0.22 μg/ml)	247	248	244	246.0	3.8	
DAPI (5.6 μg/ml), Alexa488 (0.22 μg/ml), Alexa594 (0.22 μg/ml)	81	82	84	82.0	2.5	

insulin-like growth factors 1 (IGF-1) and 2 (IGF-2) had a mild effect. Class III β-tubulin (TUBB3) was used as a neuronal marker, and nestin was used as a specific marker for adult hippocampal neural progenitor cells (Fig. 1) (Chen et al., 2007). The housekeeping protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a "loading control" and DAPI was used to represent cell numbers. To culture adult hippocampal neural progenitor cells, three-month-old Wistar rats (Hebei Medical University Experimental Animal Center) were euthanized with a lethal dose of Nembutal that was administered intraperitoneally (200 mg/kg). This protocol was consistent with the recommendations of the institutional guidelines for the care and use of laboratory animals from the Research Ethics Committee of the Second Hospital of Hebei Medical University. Next, the hippocampus was dissected and cut into small pieces in Hibernate A/2% B27/0.5 mM glutamine (Thermo Fisher Scientific) on ice, followed by digestion with 12 ml papain (2 mg/ml) (Worthington, Freehold, NJ, USA) in Hibernate A/B27 for 30 min at 30°C with shaking, and washed gently with 5 ml Hibernate A/2% B27 once. The tissue was briefly triturated in 2 ml of Hibernate A/2% B27 with a 1 ml pipette, allowed to settle for 1 – 2 min, then 2 ml of supernatant was collected. This dissociation procedure was repeated 3 times. The resulting 6 ml of cell suspension was spread over a 4 ml density gradient medium containing OptiPrep™ Density Gradient Medium (7, 9.4, 11.7, 16.4%, 1 ml each, in Hibernate A/2% B27) (Sigma-Aldrich, St. Louis, MO, USA). This was centrifuged at 800 g at room temperature for 15 min. The upper 7-ml layer of supernatant

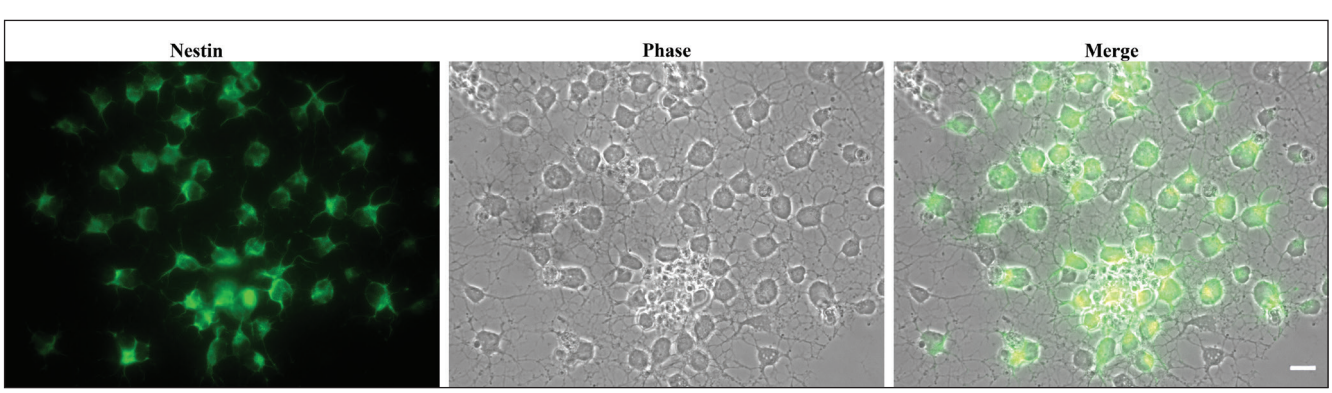

Fig. 1. Immunofluorescent staining for a cell-specific marker. This figure shows immunofluorescence with anti-nestin antibody markers for immature hippocampal neural progenitors; scale bar: 10 μm.

Table III. The OD value of single and mixed fluorescent dyes at filter set 590 nm/645 nm, gain at 50.

Fluorescent dyes	OD value			Mean	The changes (%) compared to a single fluorescent dye	Mean
DAPI (50 μg/ml)	0	0	0	0.0		
DAPI (16.7 μg/ml)	0	1	0	0.0		
DAPI (5.6 μg/ml)	0	1	0	0.0		
Alexa488 (2.0 μg/μl)	0	1	0	0.0		
Alexa488 (0.67 μg/μl)	1	0	0	0.0		
Alexa488 (0.22 μg/μl)	0	0	1	0.0		
Alexa594 (2.0 μg/μl)	416	399	392	402.0		
Alexa594 (0.67 μg/μl)	137	135	139	137.0		
Alexa594 (0.22 μg/μl)	46	45	45	45.0		
DAPI (50 μg/ml), Alexa488 (2.0 μg/ml), Alexa594 (2.0 μg/ml)	438	427	417	427.0	6.2	6.5
DAPI (16.7 g/ml), Alexa488 (0.67 μg/ml), Alexa594 (0.22 μg/ml)	146	148	145	146.0	6.6	
DAPI (5.6 μg/ml), Alexa488 (0.22 μg/ml), Alexa594 (0.22 μg/ml)	48	48	49	48.0	6.7	

was discarded. The remaining 3 ml of supernatant was collected and diluted in 5 ml of Hibernate A/2% B27 and centrifuged at 800 g for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 15 ml Neurobasal A/2% B27/0.5 mM glutamine. The cells were plated into a 100 mm Corning cell culture treated (TC) dish (Corning Incorporated Life Sciences, Tewksbury, MA, USA) pre-coated with 0.01% Poly-D-lysine (Sigma-Aldrich). This was incubated at 37°C with 5% CO_2 for 1 h. The plates were washed with warm Neurobasal A/B27 medium twice. Next, cells were cultured with culture medium containing Neurobasal A/B27/0.5 mM glutamine/100 unit/ml penicillin, 100 g/ml streptomycin (Thermo Fisher Scientific), and 10 ng/ml fibroblast growth factor 2 (FGF-2) (Thermo Fisher Scientific). The medium was changed every 2 days. FGF-2 (10 ng/ml) was used for the first two weeks, followed by 20 ng/ml for further culture. The cells were passaged at confluence by trituration with a 1 ml pipette. The third passage was used for this study.

The cells were seeded into a 96-well black polystyrene microplate (clear flat bottom, matrix active group TC-treated) (Corning Incorporated Life Sciences) pre-coated with 0.01% Poly-D-lysine at different cell densities (1×, 2×, 4×, 8× 10^4 per well) in 64 μl of culture medium with 5 ng/ml FGF-2. The cells were treated the following day with 136 μl of culture medium (with 5 ng/ml FGF-2) containing different growth factors, then cultured for 2 days. For the control cells, only the culture medium was used; for CNTF, IGF-1, and IGF-2 treated cells, the final concentration of each neurotrophic factor was 10 ng/ml; there were no cells in the blank wells. For each treatment condition, three wells of cells were used, and the experiment was repeated at least three times.

After 2 days of treatment, the cells were fixed by replacing the growth medium with 100 μl of 4% formaldehyde in Phosphate-buffered saline (PBS, 128 mM NaCl, 2 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) for 20 min. The formaldehyde solution was removed and cells were washed 3 times with 200 μl of washing buffer (0.1% Triton X-100 in 0.05 M TBS). Each washing step was performed for 5 min with gentle shaking. The washing buffer was removed, 100 μl of blocking buffer (4% normal goat serum, 0.1% Tween-20 diluted in TBS) was added, and the plates were incubated for 30 min at room temperature. The blocking buffer was replaced with primary antibodies (polyclonal rabbit antibody against neuronal TUBB3 (1:1000 dilution) (BioLegend, San Diego, CA, USA) and 6C5 monoclonal mouse antibody against GAPDH (1:500 dilution) (Thermo Fisher Scientific) diluted in the blocking buffer and incubated for 45 min at 37°C. The primary antibody was removed and the cells were washed for 3 × 5 min with 200 μl of washing buffer. The cells were then incubated in 50 μl blocking buffer containing Alexa 488-conjugated goat anti-mouse antibody (1:1000 dilution) and Alexa 594-conjugated goat anti-rabbit antibody (1:1000 dilution) for 1 h at room temperature. The secondary antibodies were removed, and the cells were rinsed 3 times for 5 min with 200 μl washing buffer and then 2 × 5 minutes with 200 μl TBS. The rabbit or mouse IgG

was used respectively as a negative control. The cells were incubated for 10 min at room temperature in 50 µl TBS containing 50 µg/ml DAPI. The DAPI solution was aspirated and the cells were rinsed 3 times for 5 min with 200 µl TBS. The 96-well plate was gently tapped onto paper towels to remove excess liquid from the wells and 100 µl TBS was added to each well. The triple-labeled fluorescence image of adult hippocampal progenitor cells is shown in Fig. 2. The FI value of three fluorescent dyes in each well was measured on the Cyto Fluor Multi-Well Plate Reader, series 4000 with the corresponding filter setting.

The results showed that the FI ratio of TUBB3 to GAPDH (TUBB3/GAPDH) in CNTF-treated cells was significantly higher than control cells under all cell density conditions. The TUBB3/GAPDH ratio in IGF-1 and IGF-2-treated cells was slightly increased under higher cell density conditions (4× and 8 × 10^4/well), although not significantly (Fig. 3A). This result is consistent with previous reports (Chen et al., 2007). The FI ratio of BIII to DAPI (BIII/DAPI) in CNTF-treated cells was significantly higher than that of control cells under the cell density of 1 ×, 2 × and 4 × 10^4/well. The ratio values of BIII/DAPI in CNTF-treated cells significantly decreased with the increase in cell density (Fig. 3B). For the FI ratio of GAPDH to DAPI (GAPDH/DAPI), there was no significant difference between treated cells, however, this ratio value decreased significantly with increased cell density (Fig. 3C). These results indicate that the CNTF-induced significant increase in BIII expression in adult hippocampal neural progenitor cells can be detected regardless of whether GAPDH or DAPI is used

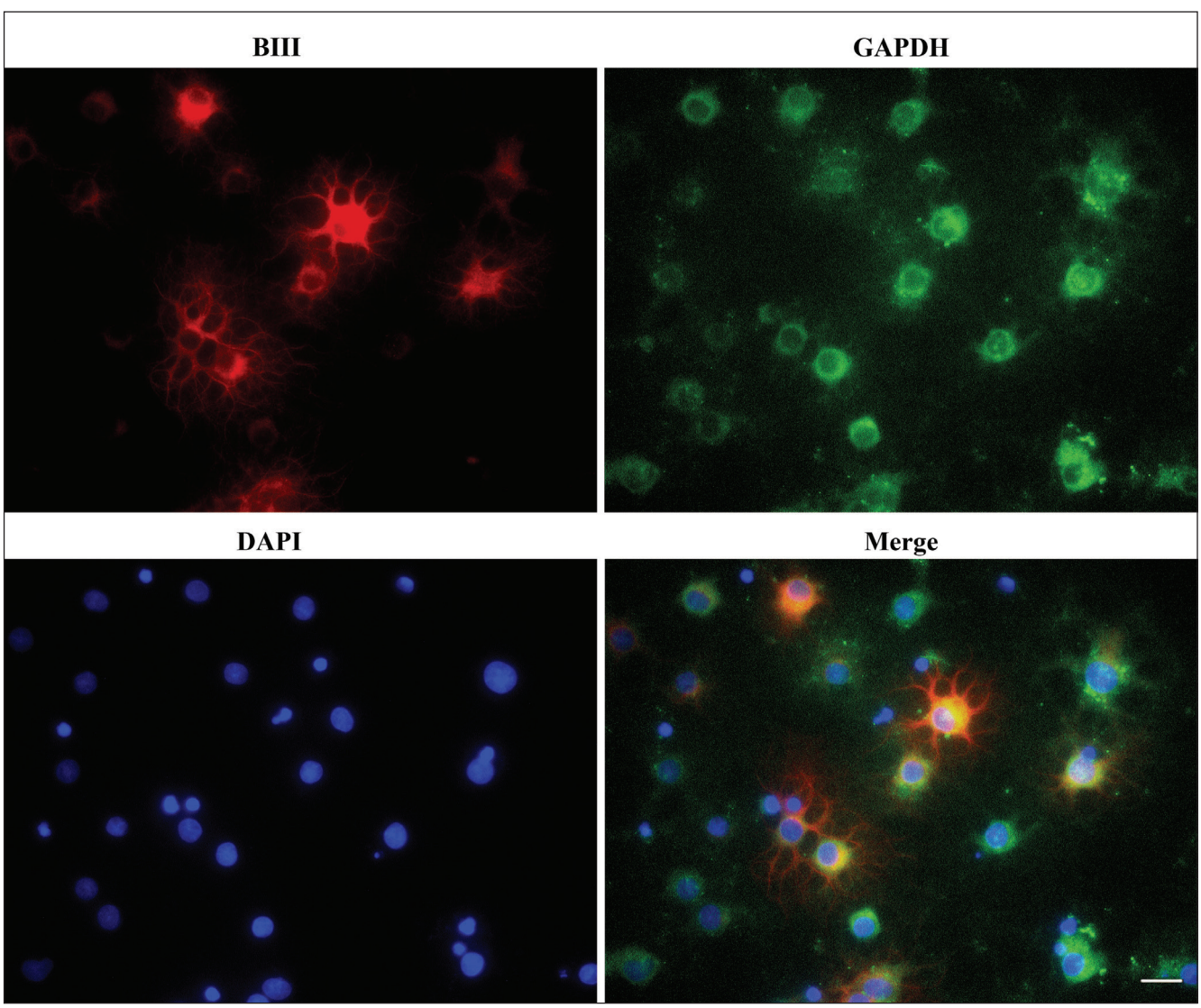

Fig. 2. Triple-labeled fluorescence image of adult hippocampal progenitor cells. This figure shows confocal immunocytochemical analysis of cells co-labeled with BIII (red), GAPDH (green), and DAPI (blue); scale bar: 10 µm.

as a reference. In the same way, our previous experiments have confirmed CNTF increased the astroglial (GFAP) level (Chen et al., 2007). However, GAPDH was better because it is less affected by cell density. Under conditions of high cell density, the growth space of the cells is limited, resulting in a decrease in the content of cytoplasmic components relative to the content of the nucleus; the proportion of the cytoplasm decrease resulted in a reduction of the absolute difference in drug action, with a nonsignificant statistical difference, which is responsible for the decrease in BIII/DAPI and GAPDH/DAPI as the cell density increases. Therefore, if DAPI is used as a reference, the cell density should be between 1× and 4 × 10^4/well.

The majority of *in vitro* studies focus on the embryonic or neonatal rat/mouse neural progenitor cell culture because adult neural progenitor cells are difficult to cultivate. However, it has been shown that adult and embryonic/newborn neural progenitor cells do not perform equally in many aspects such as electrophysiology, development, regeneration, and pathological features (Urbán and Guillemot, 2014; Zhang and Jiao, 2015). Thus, for certain age-related diseases, such as Alzheimer's disease and Parkinson's disease, the application of embryonic/newborn neural progenitors is not suitable. To simulate the physiological conditions of neurodegenerative diseases, adult hippocampal progenitor cells should be used.

Both neurosphere and adherent culture systems can be used to culture neuronal progenitor cells (Sun et al., 2011; Guo et al., 2012); however, adherent culture systems may be better for this type of cell-based fluorescence assay using a microplate. During the cultivation of adherent neural progenitor cells, there are some critical points that are worthy of attention: cell culture plate and coating: based on our experience, Corning cell culture treated (TC) dishes (100 mm) or flasks (75 cm^2) are the most suitable. Freshly prepared 0.01% poly-D-lysine should be used, the culture dish or flask coated for 3–6 h at room temperature, and then washed with 10 ml sterilized water 5 times. Further, altering the number of washing steps is not conducive to progenitor cell growth. We used 2 mg/ml papain instead of trypsin to digest the isolated hippocampal tissue because papain is gentler and does not overly digest, which ensures a stable digestion and high cell viability. We further purified neurons using OptiPrep density gradient centrifugation. Compared with traditional methods, such as simple centrifugation, cell straining, and centrifugation, OptiPrep density gradient centrifugation can effectively remove impurities, such as cell debris, oligodendrocytes, and microglia. The brain and hippocampal tissue were stored in pre-chilled Hibernate-A/B27 media instead of Hanks or PBS after dissection. The neuronal metabolism remains in progress in an ice bath; therefore, the sugar-free environment of Hanks' liquid is not conducive. The Hibernate-A/B27 medium contains a variety of nutrients, such as glucose, inorganic salts, pyruvic acid, amino acids, and vitamins, which can be used to preserve cells and to maintain the activity of cells to the maximal extent. The first generation of cell culture generally takes 3–4 weeks. At this stage, the cells are very sensitive. Except for changing

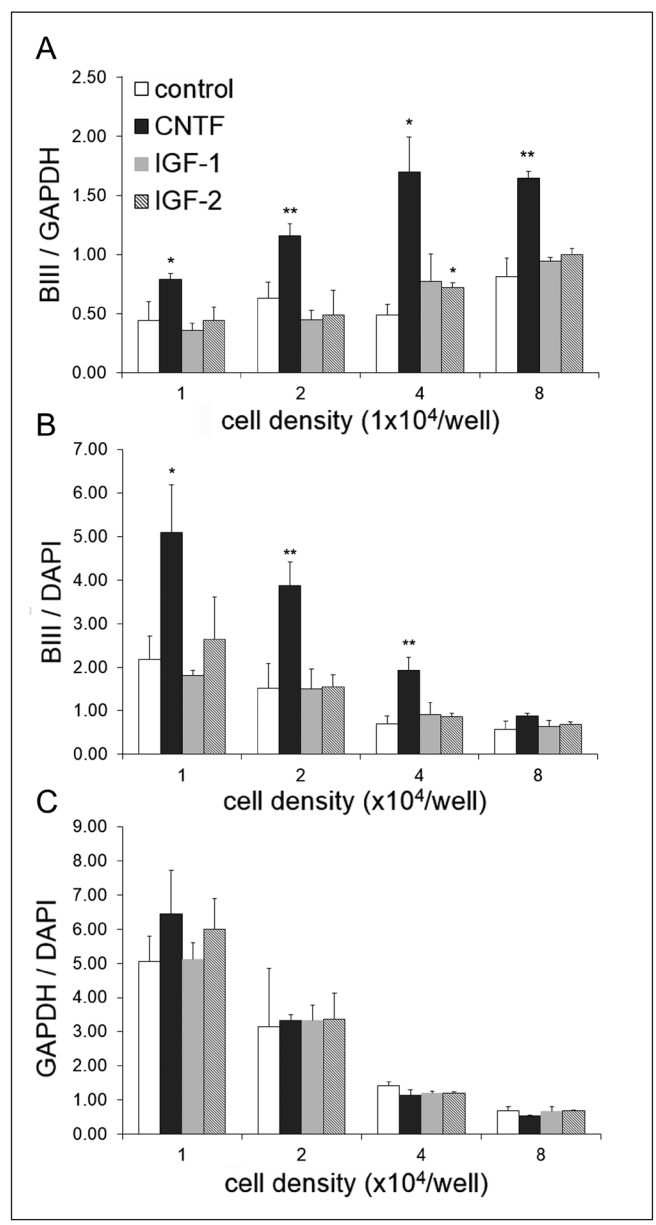

Fig. 3. Fluorescence intensity ratio of the markers used for staining adult hippocampal progenitor cells at different densities. This figure shows: (A) BIII/GAPDH in IGF-1 and IGF-2 treated cells slightly increased under higher cell density conditions; however, this was not statistically significant. (B) BIII/DAPI ratios in CNTF-treated cells significantly decreased with an increase in cell density. (C) GAPDH/DAPI significantly decreased with increased cell density. *p<0.05; **p<0.01; ***.

medium, repeated observation is not conducive to cell growth. In addition, during the immunofluorescence staining, the microplate should be rinsed as gently as possible to avoid cell detachment from the microplate.

With the development of combinatorial chemistry and diverse compound libraries, the pharmaceutical industry and academia have used high-throughput screening (HTS) technology to screen thousands of novel compounds. HTS analysis can be divided into two categories, biochemical assays, which require clear targets for drug action, such as enzymatic activity, receptor-ligand binding, ion channels, and protein–protein interactions; and cell-based assays, which are based on the actions of a drug on whole cells. Often a direct molecular target is not required; therefore, cell-based assays have become more favorable in recent years (An and Tolliday, 2010). Detection methods used in cell-based HTS assays include electrochemical methods, such as electrochemical biosensors (Liu et al., 2014); optical detection methods, such as colorimetric, luminescent, or fluorescent methods (e.g. MTT assay and cell-based ELISA) (Zang et al., 2012; Kohl and Ascoli, 2017); and high-throughput image analysis. This method uses high-throughput fluorescence and confocal microscopes with advanced analysis software (Nierode et al., 2016).

Here, we described a simple, rapid cell-based immunofluorescence assay for screening the potential of new drugs to promote neurogenesis of adult hippocampal neural progenitor cells. The reagents and instruments used in this method are inexpensive and easy to obtain, and this assay can be performed in a laboratory under general conditions. In addition, this method can use different antibodies to simultaneously label two target proteins related to neuronal differentiation, such as doublecortin, MAP2, NeuN, neurofilaments (e.g. NF-L and NF-M), neurotransmitter synthesizing enzymes (e.g. GAD and TH), and synaptic proteins (e.g. PSD-95, synaptophysin, and VAMP). The third marker is DAPI, which serves as a reference for the number of cells to reduce the variation caused by the number of cells in a culture well. Therefore, this method could be a powerful tool for the rapid screening of drugs that promote adult neurogenesis.

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