BTX Applications

Tech-Trends
Volume 3, Series 2

High content analysis of primary postnatal cortical neurons

High content screening of cortical neurons identifies novel regulators of axon growth

Background:

Neurons in the central nervous system lose their capacity for axon regeneration as they mature, and it is widely hypothesized that changes in gene expression are responsible. Testing this hypothesis and identifying the relevant genes has been challenging because hundreds to thousands of genes are developmentally regulated in CNS neurons, but only a small subset are likely relevant to axon growth. Here we used automated high content analysis (HCA) methods to functionally test 743 plasmids encoding developmentally regulated genes in neurite outgrowth assays using postnatal cortical neurons.

Methods:

We obtained a library of nearly 16,000 mammalian expression vectors (Open-Biosystems), which contained plasmid DNA corresponding to 445 of our candidate genes represented redundantly by 743 clones. To screen these hundreds of candidate genes we adopted the strategy of electroporation in 96-well format, based on (Buscher et al., 2006), which allows many genes to be tested in a single experiment.

Results:

We established conditions for transfecting cortical neurons with about 50% efficiency, as assessed by expression of EGFP and mCherry fluorescent reporters (Supplemental Fig. 1). Although relatively high, 50% transfection efficiency still requires some means to specifically identify transfected neurons for downstream analysis. Thus we optimized a strategy of co-transfection with a fluorescent reporter plasmid. Three days after transfection with pCMV-SPORT6 GFAP, we detected GFAP expression in 34% of neurons. When GFAP was co-transfected with mCherry plasmid at a ratio of about 6:1, more than 80% of mCherry-positive neurons also expressed GFAP.

Conclusion:

By combining electroporation in 96-well format with automated image analysis and streamlined data analysis, we could comfortably test around 50 plasmids and acquire detailed morphological data from around 50,000 neurons per week. We indentified both growth inhibitors (Ephexin, Aldolase A, Solute Carrier 2A3, and Chimerin), and growth enhancers (Doublecourtin-like, Kruppel-like Factor 6, and CaM-Kinase II gamma), some of which regulate established growth mechanisms like microtubule dynamics and small GTPase signaling.

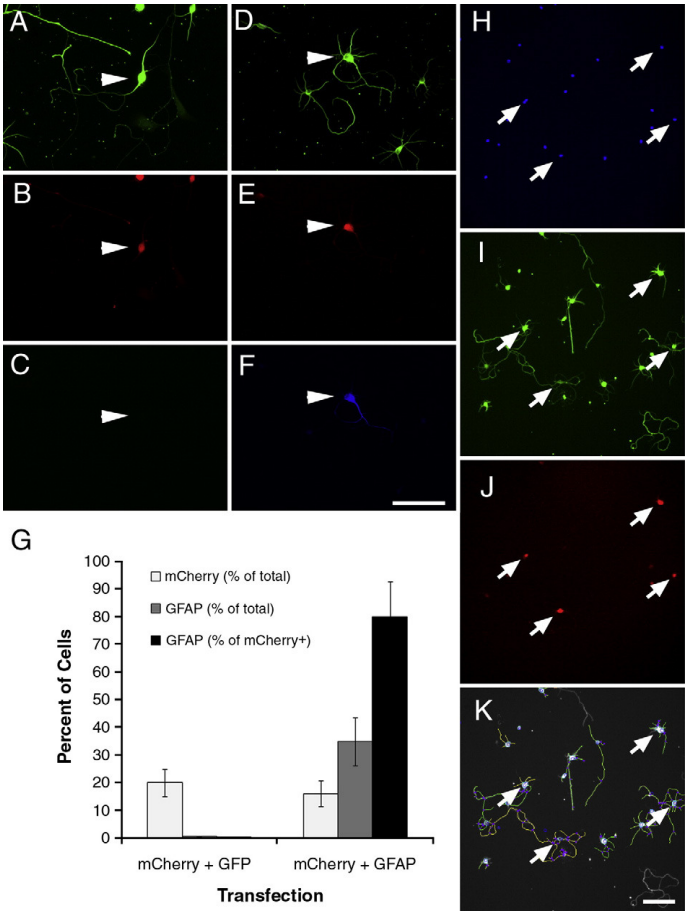


Fig. 1. Cortical neurons transfected in 96-well format are identified by co-transfected mCherry reporter and efficiently traced by Cellomics instrumentation. (A–G) P1 cortical neurons were co-transfected with mCherry and either EGFP or GFAP. (A–C) Control neurons transfected with mCherry and EGFP express βIII tubulin (arrowhead, A) and mCherry (arrowhead, B), but do not express GFAP (arrowhead, C). (D–E) Neurons transfected with mCherry and GFAP express both mCherry (arrow, E) and GFAP (arrow, F). (G) Quantification shows that 80% of mCherry-positive neurons express GFAP after co-transfection. More than 300 neurons were quantified per condition. (H–K) P1 cortical neurons transfected with mCherry were cultured for 3 days prior to fixation, staining, and automated scanning (Kineticscan, Cellomics). Only cells identified as neurons by βIII tubulin staining (I), and successfully transfected with mCherry reporter (arrows, J) were included in subsequent analysis. (D) Digitized overlay shows effective tracing of cell processes. Scale bars, 100 μm.

BTX®
HARVARD APPARATUS
Molecular Delivery Systems

84 October Hill Rd. Holliston, MA 01746 Toll Free Ph: 800-272-2775 or 508-893-8999
Email: btxinfo@harvardapparatus.com Web: www.BTXonline.com

Cell Preparation:

Postnatal rats (P1 or P5) were sacrificed by decapitation and the brains placed in ice-cold Hibernate E (minus $CaCl_2$) (Brainbits #HE-Ca 500). Meninges were removed. Frontal cortex was isolated, minced finely with a razor blade, and incubated in 10 ml dissociation media (Hibernate E containing 20 U/ml papain, Worthington) and 150 µg/ml DNAse (Sigma #5025), and incubated for 30 min at 37ºC with constant shaking. Cell clumps were rinsed by pelleting (10xg, 10 min) and resuspended in Hibernate E+2% B27, then pelleted, resuspended in 1.5 ml Hibernate E+2% B27, and gently triturated three times with a lightly fire polished pipette. Remaining cell clumps were pelleted and resuspended in 10 ml Hibernate E containing 25% trypsin (Gibco #15090 and 150 µg/ml DNAse). Cells were incubated for 30 min at 37ºC with constant shaking, then pelleted and resuspended in 5 ml Hibernate E+2% B27. Cells were then sequentially triturated by resuspending in 1.5 ml Hibernate E+2% B27, triturating three times with a lightly fire polished pipette, then letting cell clumps settle for two minutes. The supernatant was removed to a separate collection tube, and the process repeated until no cell clumps were visible. Typically a total of 12-14 mls of cell suspension containing a total of 8-10 million cells was collected. Dissociated neurons were pelleted and resuspended in Internal Neuronal Buffer (INB) (KCL 135 mM, $CaCl_2$ 0.2 mM, $MgCl_2$ 2mM, HEPES 10 mM, EGTA 5 mM, pH 7.3) at a concentration of 2 x 10^6/ml.

Table 1
Hit genes do not affect cell survival. P5 cortical neurons were untransfected (no plasmid), or transfected with EGFP (control) or with hit genes, prior to culture for 3 days and measurement of cell death using Sytox orange. 95% of neurons cultured without supplements died (***$p<.001$, ANOVA with post-hoc Dunnett's). Transfection efficiencies were above 50% in all experiments. No hit genes significantly altered cell survival compared to controls ($n>1000$, $N=3$, ANOVA with post-hoc Dunnett's).

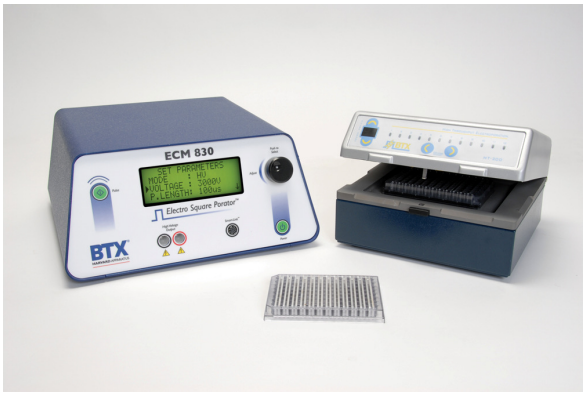
Gene	% survival
No transfection	51.4 (±5.6)
No supplements	5.6 (±1.8)***
EGFP	46.9 (±5.6)
ALDOA	47.3 (±5.8)
CAMK2G	46.8 (±8.2)
CHN1	46.4 (±7.6)
DCL	46.2 (±7.9)
DCX	47.1 (±6.1)
KLF6	43.7 (±5.7)
NGEF	45.6 (±5.8)
SLC2A3	44.7 (±7.2)

Electroporation Settings:

Choose Mode:	LV
Set Voltage:	350 V
Electrode Gap:	2 mm
Set Pulse Length:	300 µs
Set Number of Pulses:	1
Desired Field Strength:	1750 V/cm

Electroporation Procedure:

Total Volume:	25 µl
Transfectant conc:	0.5 µg mCherry and 3 µg plasmid
Cell density:	2 x 10^6/ml
Pulse:	Press Automatic **Start** to activate Charge and Pulse
Post Pulse Treatment:	100 µl of Hibernate E with 2% B27 supplement was added to each well to aid cell recovery, and cells were transferred to 96-well plates (1600 cells/well).



450421 Includes ECM 830 Generator, 4 mm gap, 2 x 96-Well Plates, Plate Seals and HT-200 Plate Handler

450450 96-Well Disposable Electroporation Plate, 2 mm gap, 125 µl, 1 plate

References:
Murray G. Blackmore[a,*], Darcie L. Moore[b,d], Robin P. Smith[a,b], Jeffrey L. Goldberg[b,d], John L. Bixby[a,b,c], Vance P. Lemmon[a,b,c]
[a]The Miami Project to Cure Paralysis, Dept. of Neurological Surgery, University of Miami. [b]Neuroscience Program, University of Miami. [c]Dept. Of Pharmacology, University of Miami. [d]Bascom Palmer Eye Institute, Miller School of Medicine, University of Miami.
doi:10.1016/j.mcn.2010.02.002

Molecular and Cellular Neuroscience 44 (2010) 43-54

BTX® HARVARD APPARATUS Molecular Delivery Systems