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Automated Cell Plating and Sample Treatments for Fixed Cells in High Content Assays

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Summary

Robust and reliable methods for the manipulation of neural cell lines, by passaging, plating, dye labeling, imaging, fixation, and immunocytochemistry, are required to enable consistent, reproducible screens to be performed. We describe herein procedures and processes we have established to maximize the level of consistency of cell plating, fixation, and dye or antibody labeling, to ensure that assays which we are running on a routine basis remain consistent across long periods of time. These procedures involve a variety of fully or semiautomated steps, using high-quality commercially available liquid handling and dispensing technology.

Key Words: Cell plating; chemokinesis; fluorescence imaging; neurospheres; neurite outgrowth.

1. Introduction

Much attention in the high content screening field has been applied to optimizing the quality of image analysis to maximize value and information *(1,2)*. Equally important; however, is ensuring a high standard, and a high level of consistency, in the preparation of cells for analysis. This is particularly important for screening assays, which run on a week-by-week basis, whereby variability between plates and across weeks will critically affect the utility of an assay and the interpretability of the results. We have established procedures and processes to maximize the level of consistency of cell plating, fixation, and dye or antibody labeling, to ensure that high content assays which run on a routine basis remain reliable across long periods of time. These procedures involve fully and semiautomated steps, and we describe the high-quality commercially available liquid handling and dispensing technology we have used to implement these approaches.

2. Materials

2.1. Automated Cell Plating

1. Clonetics human neural progenitor cells.
2. Plating media (*see* **Subheading 2.2.1.**, **step 5**).
3. 50- and 15-mL conical tubes.
4. Phosphate-buffered saline (PBS) Ca/Mg free.
5. Accutase.
6. Hanks' buffered saline solution (HBSS).
7. Trypan blue.

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2.2. Sample Treatments for Fixed Cells

2.2.1. An Automated Chemokinesis Assay

1. Sterile PBS and PBS pH 7.4.
2. Blacksided clear bottomed 96-well plates of cells.
3. Cellomics® (Pittsburgh, PA) Cell Motility Hit Kit.
4. 5% Paraformaldehyde in PBS: stored at 4°C.
5. Sterile plating medium: Dulbecco's modified Eagle's medium (DMEM)/F12 3/1, transferrin 50 µg/mL, insulin 5 µg/mL, progesterone 20 n*M*, putrescine 100 µ*M*, T3 30 n*M*, and selenium 30 n*M*.
6. Positive control and test compounds.
7. Bovine serum albumin (BSA).
8. Normal goat serum (NGS).
9. Triton X-100.
10. Monoclonal anti β-tubulin antibody (Sigma, St. Louis, MO).
11. Alexa-594 goat antimouse secondary antibody (Molecular Probes, Eugene, OR).
12. 2X Blocking buffer prepared freshly and stored at 4°C until required: PBS containing 0.2% Triton X-100, 10% NGS, and 2% BSA.
13. 2X Primary antibody solution prepared freshly and stored at 4°C until required: PBS containing 10% NGS, 2% BSA, and 1/500 primary antibody.
14. 2X Secondary antibody solution prepared freshly and stored at 4°C until required. (This solution is light sensitive.) PBS containing 10% NGS, 2% BSA, and 1/1000 secondary antibody, and 60 µ*M* Hoechst 33342.
15. Cellomics Arrayscan II and Cellomics Cell Motility Algorithm.

2.2.2. Compound Incubation and Plate Processing of a Neurite Outgrowth Assay

1. Sterile PBS and PBS pH 7.4.
2. Black-sided clear-bottomed 96-well plates of cells.
3. Positive control and test compounds.
4. BSA.
5. NGS.
6. Triton X-100.
7. Ice-cold methanol.
8. Primary antibodies as appropriate to assay.
9. Secondary antibodies as appropriate to assay.
10. Hoechst 33342.
11. 2X Blocking buffer.
12. 2X Primary antibody solution.
13. 2X Secondary antibody solution.
14. Automated liquid handling and plate washing.
15. High content screening plate imager.

2.2.3. Fully Automated Single Cell Kinetic and Immunocytochemical Assay

1. KHB (Krebs-Henseleit-bicarbonate buffer) pH 7.4.
2. PBS pH 7.4.
3. Black-sided clear-bottomed 96-well plates of cells.
4. Test compounds.
5. Fluo 3-AM.
6. Pluronic F-127.
7. 2X Fluo3-AM dye solution (This solution is light sensitive.) KHB containing 8 µ*M* Fluo 3-AM and 0.16% Pluronic F-127.
8. BSA.
9. NGS.
10. Triton X-100.
11. Ice-cold methanol.
12. Primary antibodies as appropriate to assay.

13. Secondary antibodies as appropriate to assay.
14. Hoechst 33342.
15. 2X Blocking buffer.
16. 2X Primary antibody solution.
17. 2X Secondary antibody solution.
18. Automated liquid handling, plate processing, and imaging on integrated robotic platform.

3. Methods

3.1. Automated Cell Plating

Clonetics human neural progenitor cells (Cambrex, NJ), growing as neurospheres, were treated with enzymes to create a single cell suspension, counted, and diluted to a predetermined number, and plated into 96-well plates using a Multidrop (Thermo Electron Corporation, Waltham, MA).

3.1.1. Generation of a Single Cell Suspension

1. Spheres were transferred into a 50-mL conical tube with a minimal volume of media, allowed to settle, the liquid removed, and the spheres washed twice with 50 mL PBS (*see* **Note 1**).
2. Following removal of the last wash of PBS, 5 mL of one-fifth dilution of Accutase in HBSS was added and the tube placed in a vigorously shaking water bath at 30ºC for 30 min (*see* **Note 2**).
3. Cells were triturated using a 1000 µL pipet tip until most clumps were dispersed (10–15 times), 40 mL PBS added and the cell suspension centrifuged at 150 rcf for 5 min.
4. Supernatant was removed, 2 mL plating media added, and the cells triturated again.
5. The suspension was then transferred to a 15 mL conical tube, 6 mL plating media added, and centrifuged at 10 rcf for 1 min.
6. The supernatant was collected and the pellet discarded (*see* **Note 3**).
7. The cell suspension was counted using the Trypan blue exclusion method on a Cedex cell counter (Innovartis) (*see* **Note 4)** and diluted appropriately.

3.1.2. Plating (see ***Note 5***)

1. To ensure sterility of the cultures, a multidrop was located in a biological safety cabinet and sprayed with 70% ethanol, with the tubing primed with 70% ethanol followed by sterile PBS.
2. The cell suspension was primed through the tubing until air bubbles were eliminated and then each plate was processed in turn.
3. The cell suspension was mixed by swirling between each plate. When the pipeting was complete, the plates were transferred to a cell culture incubator (humidified at 37ºC with 5% CO_2) (*see* **Note 6**).

3.2. Sample Treatments for Fixed Cells

3.2.1. An Automated Chemokinesis Assay

1. Cell suspensions for this assay were plated into 96-well plates precoated with blue fluorescent beads from the Cell Motility HitKit. During incubation, moving cells displace or phagocytose the fluorescent beads leaving dark "tracks."
2. At an appropriate time-point, cells were fixed and fluorescently labeled, and the tracks quantified using a Cellomics Arrayscan II (Pittsburgh, PA). The assay described here was designed to screen for compounds, which increase the motility of human neural precursor cells (Clonetics®, Cambrex, East Rutherford, NJ) ***(3)***, but could equally be applied to investigate inhibitors of cell motility, by adding a known stimulant to the plating medium.

3.2.1.1. Assay Plate Preparation

1. 96-well black-sided microtiter poly-D-lysine coated plates (Biocoat™, BD Biosciences, Oxford, UK) were coated with laminin 1 µg/mL (*see* **Note 7**).
2. For each 96-well plate, one vial of blue fluorescent beads from the Cellomics Cell Motility Hit Kit was resuspended by vortexing for 30 s then centrifuged for 1 min at 20,000*g*.
3. Supernatant was removed, and 0.5 mL PBS was added, before vortexing and centrifugation. This washing step was then repeated.

4. The resulting bead suspension was added to 7.5 mL PBS and vortexed for 1 min (*see* **Note 8**).
5. 75 µL of bead suspension was then added to each well of the laminin-coated 96-well plate using a multichannel pipetor.
6. The plate was incubated for 1 h at 37°C and 5% CO_2, then washed very gently five times using sterile PBS and a multichannel pipetor, leaving a residual volume of 100 µL per well.
7. Plates were wrapped in foil and stored at 4°C until required.

3.2.1.2. Chemokinesis Assay

1. Test compounds were prepared at 2X[final] in plating medium containing 0.2% serum, and 50 µL added to the test wells in the bead-coated plates. 5% serum was used as a positive control.
2. Assay plates were prewarmed to 37°C and 2000 cells/well in 50 µL of plating medium were added using a Multidrop (*see* **Note 9**).
3. Plates were incubated for 18 h at 37°C with 5% CO_2.
4. All the following incubations were performed at room temperature, in the dark, to prevent photobleaching of the beads.
5. Cells were fixed by adding 100 µL/well of prewarmed 5% paraformaldehyde for 10 min at room temperature without removing the medium. Addition and washing steps were performed with a multichannel pipet and a Thermo Wellwash (Thermo Electron Corporation, Waltham, MA) plate washer.
6. Plates were washed three times with 200 µL/well PBS with a residual volume of 50 µL/well.
7. 50 µL of 2X blocking buffer was added to each well and incubated for 1 h.
8. 100 µL/well of 2X primary antibody solution was added without washing, and incubated for 1 h.
9. Plates were washed three times with 200 µL/well PBS with a residual volume of 50 µL/well.
10. 50 µL of 2X secondary antibody solution was added and incubated for 1 h.
11. Plates were then washed three times with 200 µL/well PBS and a residual volume of 200 µL/well, then sealed with thin adhesive plate seals (*see* **Note 10**).
12. Chemokinesis was quantified in an automated manner using a Cellomics ArrayScan II and Cellomics Cell Motility algorithm, acquiring nine fields per well with a ×5 objective. The algorithm outputs, which were found to be most useful for analysis were the average track area and the average number of tracks per field.

3.2.2. Compound Incubation and Plate Processing of a Neurite Outgrowth Assay

The methods described here detail the procedures used to perform a screen for promoters of neurite outgrowth in human neural precursor cells, although the general methodology and principles could be adapted to a range of fixed endpoint high content screening assays ***(4,5)***. The equipment used for processing and reading plates were a Multimek (Beckman Coulter, Fullerton, CA), a PlateTrak equipped for 96-channel pipeting and plate washing (Perkin Elmer, Boston, MA) and a Cellomics Arrayscan II; a range of suitable alternatives are available.

3.2.2.1. Compound Addition

1. Test compounds and positive and negative controls were prepared in deep 96-well microplates such that subsequent addition of 200 µL of sterile PBS on the Multimek would produce a solution with 10X final concentration. For this assay the positive control was 100 ng/mL platelet-derived growth factor (PDGF) final concentration, sterile PBS was used as the negative control and test compounds were screened at 1 µ*M*.
2. 96-well black-sided microtiter poly-D-lysine coated plates (Biocoat) were coated with 1 µg/mL laminin.
3. Cells were plated at 17,500 cells/well in 200 µL of plating medium using a Multidrop (*see* **Subheading 2.2.1.** and **Note 11**).
4. Following incubation for 1 h at 37°C with 5% CO_2, a Multimek program was used to dilute the compounds in 200 µL of sterile PBS and to immediately add 22 µL to a cell plate to produce the correct final desired concentrations (*see* **Note 12**).
5. Cell plates were incubated for 48 h at 37°C with 5% CO_2. The assay was typically performed on batches of 20 cell plates.

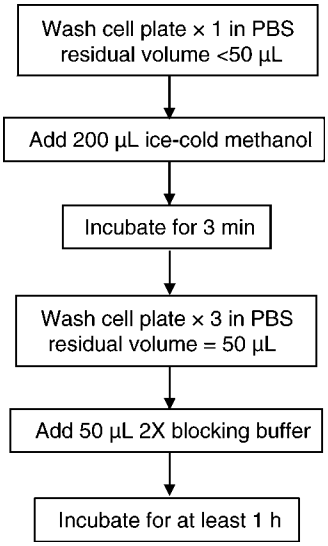

Fig. 1. A flow diagram representing the steps of the PlateTrak program used for fixing and blocking cells.

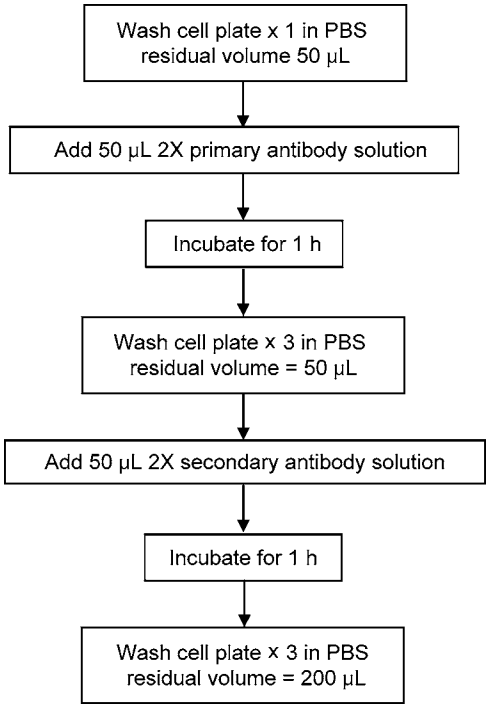

Fig. 2. A flow diagram representing the steps of the PlateTrak program used for applying and incubating primary and secondary antibodies with cells.

3.2.2.2. Cell Fixing and Labeling

1. Cell plates were fixed and antibody-labeled using a PlateTrak configured with two plate stackers, a 96-well plate washer, a 96-channel dispense head, and an autoreplenish reservoir. The cell washer reservoir was filled with PBS. A bottle of methanol standing in a bucket of ice was connected to the autoreplenish reservoir such that ice-cold methanol was continuously recycled to the reservoir during cell fixing.

2. For convenience, plate processing utilized two PlateTrak programs: one to fix cell plates and to add blocking buffer (*see* **Fig. 1**), and another to perform antibody labeling (*see* **Fig. 2** and **Note 13**).
3. Following this PlateTrak processing, cell plates were sealed using thin adhesive seals, wrapped in foil and stored at 4°C until they were read.
4. Antibodies were used at 1/1000 final concentration and typically included polyclonal or monoclonal anti-β-III tubulin (Covance), monoclonal antiGFAP (Sigma), Alexa-488 conjugated goat anti-mouse or anti-rabbit, and Alexa-594 conjugated goat anti-mouse or anti-rabbit anti-bodies (Molecular Probes) (*see* **Note 14**).

3.2.2.3. Cell Plate Imaging

1. Neurite outgrowth was quantified using a Cellomics Arrayscan II and Cellomics Neurite Outgrowth algorithm.
2. A Twister (Caliper Life Sciences, Mountain View, CA) attached to the ArrayScan allowed batched of up to 20 plates to be read automatically.
3. Satisfactory results were achieved by adapting with the Cellomics neurite outgrowth algorithm using a ×5 objective, with nine fields imaged per well. The algorithm outputs, which were found to be most useful were Neurite Outgrowth Index, and several indicators of neurite length and branching.
4. Plate data was accepted as valid if the positive control had produced at least a twofold increase in neurite outgrowth, length, and branching.
5. Compound results were normalized to positive control values to allow for inter-experiment variability, with active compounds defined as those producing increases more than 35% of control in all three parameters.
6. The images from wells with active compounds were viewed to confirm that labeling and image analysis algorithms had been applied correctly.
7. The antibody labeling produced using this automated methodology was consistent to such a degree, which identical image capture exposure times were used across an approx 8 mo screening period.

3.2.3. Fully Automated Single Cell Kinetic and Immunocytochemical Assay

The methods described here detail the procedures used to perform a kinetic signaling assay to measure dynamic changes in intracellular free calcium in individual human neural precursor cells in response to compound treatment. This was combined with posthoc immunocytochemistry to separately characterize and compare with the kinetic responses from specific subsets of cells, for example, precursor, glial, and neural cells within a mixed neural cell population ***(6–8)***. The robotic platform used to perform this assay consists of a CRS Catalyst 5 robotic arm (Thermo, San Jose, CA) integrated with an Atto PathwayHT confocal imager (now marketed as BD PathwayHT Bioimager) ***(6,7)*** fitted with a one channel pipettor head for online compound addition (BD Biosciences), a PlateTrak equipped for 96-channel pipeting and plate washing as described in **Subheading 3.2.2.** and a Heraeus Cytomat CO_2 incubator (Kendro, Bishop's Stortford, UK). The robot protocol is outlined in a flow diagram (*see* **Fig. 3**). This general methodology can be adapted to a range of combined kinetic and immunocytochemical assays in high-content single cell imaging.

3.2.3.1. Cell and Compound Plates Preparation

1. 96-well black-sided microtiter poly-D-lysine coated plates (Biocoat, BD Biosciences) were coated with 1 µg/mL laminin.
2. Cells were plated at 17,500 cells/well in 200 µL of medium using a Multidrop and incubated at 37°C with 5% CO_2.
3. A 2.5 µL of test compounds at 2 m*M* concentration was plated into 96-well microplates such that subsequent addition of 97.5 µL of KHB buffer on the Multimek (Beckman Coulter) would produce a solution at 5X final. For this assay, the positive control was 10 µ*M* methacholine, KHB buffer was used as the negative control and test compounds were assayed at 10 µ*M* (final).
4. Compound plates were lidded after dilution until ready for use.

3.2.3.2. Dye Loading, Cell Fixing, and Labeling

1. Cell plates were washed and loaded with Fluo 3-AM for live cell kinetic imaging, then fixed and antibody labeled using the PlateTrak component of the integrated system (*see* **Note 15**).

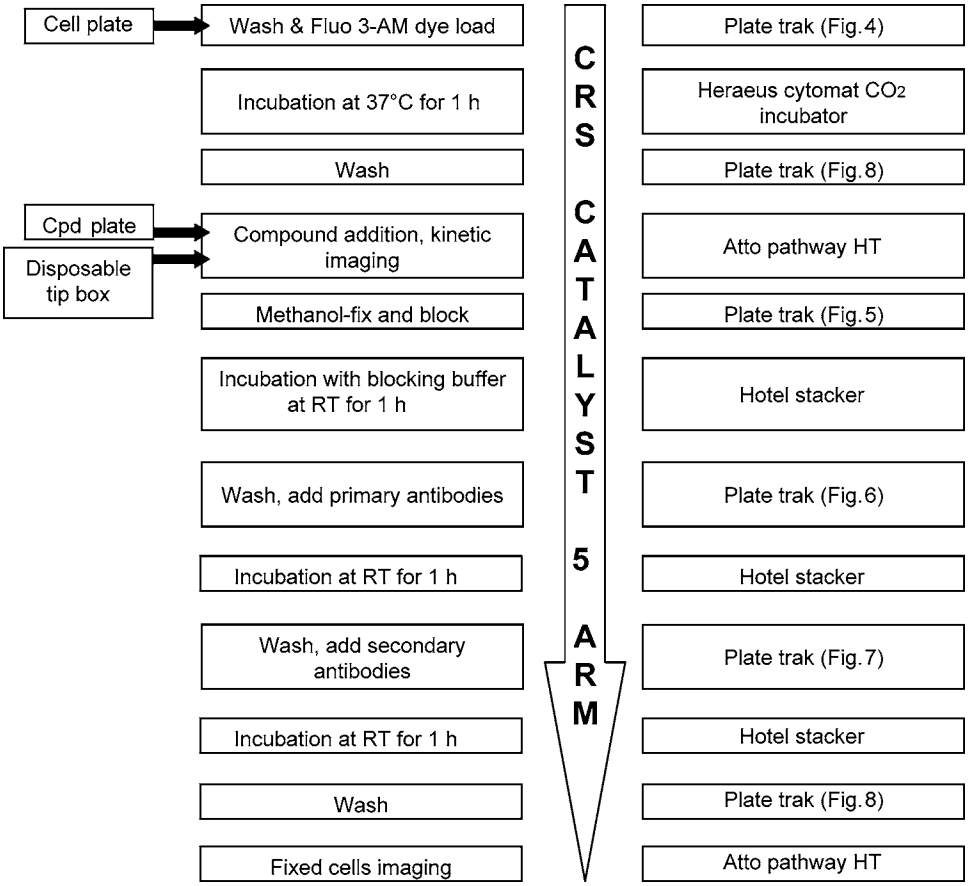

Fig. 3. Robot protocol for kinetic and endpoint combined assays on PathwayHT.

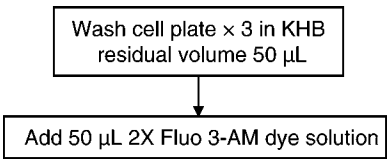

Fig. 4. Dye-loading protocol for live cell kinetic imaging on PathwayHT.

2. The PlateTrak was setup with the cell washer reservoir filled with KHB and the tip wash reservoir filled with dH_2O.
3. 2X Fluo 3-AM dye solution was placed in a foil-wrapped bird-feeder bottle connected to an autoreplenish reservoir (*see* **Note 16**).
4. A bottle of methanol standing in a bucket of ice was connected to another autoreplenish reservoir as described in (**Subheading 3.2.2.**) such that ice-cold methanol was continuously recycled to the reservoir during cell fixing.
5. Separate PlateTrak programs were executed within the robot protocol for dye loading, cell fixing, and blocking, primary and secondary antibody labeling, and cell washing (*see* **Figs. 4–8**, respectively).

3.2.3.3. Compound Addition and Kinetic Imaging

1. Each cell plate was imaged on the BD (formerly Atto) PathwayHT one well at a time.
2. Single cells were automatically identified and marked as an individual region of interest (ROI) by intensity threshold (*see* **Note 17**).

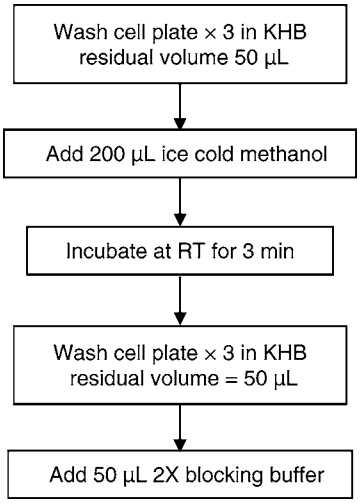

Fig. 5. Flow diagram representing the steps of the PlateTrak program used for fixing and blocking cells.

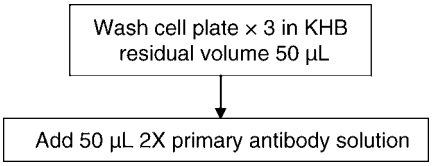

Fig. 6. Primary antibody-labeling procedure.

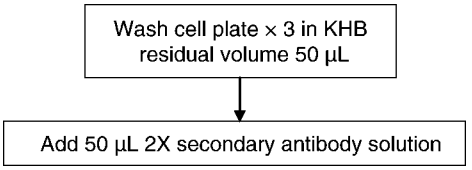

Fig. 7. Secondary antibody-labeling procedure.

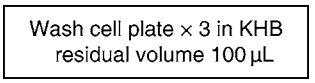

Fig. 8. Cell washing step.

3. Fluorescence in each ROI was then tracked throughout the experiment. Following 10 s of basal read, 25 µL of compounds from the 5X (final) compound plate was added to the well with 100 µL residual volume by the Atto PathwayHT one channel pipetor head, after which images were acquired for a further 60 s to capture the acute response. To ensure quality of data, the methacholine positive control peak and plateau response on each plate must fall within the normal expected amplitude range.

3.2.3.4. Posthoc Fixed Cell Imaging

1. After kinetic imaging on the BD PathwayHT, the cell plate was moved from the imaging system manually or by robotic arm and fixed and antibody labeled on the PlateTrak.
2. Monoclonal antiGFAP (Sigma), monoclonal antiTuj1 (Covance, Princeton, NJ) and monoclonal antinestin (Chemicon, Temecula, CA) primary antibodies were used at 1/1000, 1/500 and 1/500 (final), respectively.

3. Alexa-488 conjugated goat antimouse (Molecular Probes) secondary antibody was also used at 1/500 (final).
4. An image was then acquired sequentially in both Hoechst (blue) and Alexa-488 (green) channels of each well on the BD PathwayHT (*see* **Note 18**).
5. These two images were then combined to produce a composite image offline, which represented the group of cells that corresponded to the ROIs initially imaged during the kinetic experiment. This allowed users to identify, for example, the GFAP+ or Tuj1+ or nestin+ cells/ROIs, and extract their kinetic responses from which of the whole population.
6. Each well that was imaged resulted in output of a text file containing the raw fluorescence intensity data for each ROI at each time-point.
7. For analysis, text files from the kinetic experiment were exported into an inhouse Excel template, which normalized each data point to fluorescence intensity change over basal (Microsoft, Seattle, WA).
8. The normalized data for the ROIs representing GFAP+ or Tuj1+ or nestin+ cells were then exported to Graphpad Prism in which the peak response, time to reach peak response and area under curve were calculated and compared between each cell type for each compound treatment (Graphpad Software, San Diego, CA).
9. With the use of automation, assay steps such as cell washing, dye loading, and antibody labeling were standardized. This helped to reduce experimental variability and improve consistency of plate handling.
10. Liquid handling equipment, such as the Multidrop, Multimek, and PlateTrak are tested regularly to make sure their dispense volume and CVs fall within the manufacturer's specifications.

4. Notes

1. The contamination of cell cultures by *Mycoplasma* can remain undetected and yet cause effects on cell growth, cytokine production, DNA/RNA synthesis and so on. *Mycoplasma* can be detected using proprietary kits, and infected cell cultures treated with antibiotics or discarded.
2. It is preferable, wherever possible, to avoid the used of proteolytic enzymes when generating cells for an assay. There are a number of nonenzymatic cell dissociation solutions, which are very effective for cell lines growing as monolayers.
3. Sieves of 100 or 70 μM are an alternative to the final centrifugation step (Beckton Dickinson).
4. Hemocytometers are the traditional method of cell counting and could be used but may introduce more variation. Cell counters such as the Cedex provide a viable cell count per mL and a measure of whether the cell suspension was single cell.
5. It is important to minimize well-to-well and plate-to-plate variation. This can be achieved by steps as simple as using an eight-channel pipet rather than a single channel, and ensuring adequate mixing of the cell suspension. For larger plate numbers we use a Multidrop or similar liquid handler, these in turn can be semiautomated with Twister arms, or plate preparation can be performed on fully automated using systems such as SelecT® (The Automation Partnership, UK). SelecT is a Stäubli arm-controlled cell culture system, which can generate flasks and assay plates using an integrated Cedex cell counter to determine viability and seeding density. We use SelecT for the cell culture of robust, adherent cells, which form the majority of our cell lines: it is able to generate a very consistent, high quality cell output and, in our hands, has proven to be very reliable and capable of working 24/7. The advantages of using an automated system include the consistency of product, the ability to plates cells every day of the week and at specific time points in the day. SelecT is best suited to adherent cell lines such as 293, CHO, or SHSY5Y but it can be used for suspension cultures which can be passaged by dilution. Whatever liquid handling equipment is chosen to generate plates it is very important to ensure it is calibrated regularly: we check the calibration of our Multidrops weekly and change the tubing monthly. In addition once a week we clean the tubing by priming, and leaving for 1 h in a bleach solution to remove protein buildup (trypsin is an acceptable alternative) then washing with copious water.
6. Some cell types tend to settle towards the edges of the plate leading to an uneven density. This is particularly prevalent if the incubator has a vibration. If this occurs, a simple fix is to leave the plates for 1 h on the lab bench after pipeting before placing in the incubator.
7. Laminin was determined to be optimum substrate for this cell type in separate experiments. For alternative cell types the appropriate substrate would need to be investigated.

8. It is possible to prepare the bead suspension at half of the manufacturer's recommended concentration to reduce costs; however, this increases the fragility of the bead layer and the likelihood of assay failure.
9. Cell viability was assessed in parallel experiments, by preparing identical test compounds in standard 96-well laminin coated plates and plating cells at a variety of densities. Following 18 h incubation at 37°C with 5% CO_2 a staining solution comprising 30 μ*M* Hoechst 33342, 5 μ*M* calcein-AM, and 2 μ*M* propidium iodide (all from Molecular Probes) was added to the plates, to indicate nuclei, live cells, and dead cells, respectively. Following a further 30 min incubation cells were imaged and quantified using a Cellomics Arrayscan II and Cellomics Cell Viability algorithm, acquiring nine fields per well with a ×10 objective. With the human neural precursor cells, 2000 cells/well was determined to be a suitable density to use for minimizing contact-inhibition of chemokinesis while maintaining cell viability and convenient image-analysis.
10. Methanol fixing was found to be unsuitable for this assay, as cells were prone to detaching. PFA fixing times were varied between 10 min and 1 h, with no difference in antibody labeling intensity. All solution addition and washing steps were performed manually with a multichannel pipet and a Thermo Wellwash plate washer. These steps could be automated to increase the throughput of the assay, but for sensitive cell types like neural cells, care is required in defining the pipeting and washing heights and speeds. Alexa-594 conjugated phalloidin was investigated as a cytoskeletal marker, but the labelling intensity was much less than using primary, and secondary antibodies as described.
11. The plating medium and cell density are critical factors, which must be determined empirically before performing an assay of this nature. The plating medium has to sustain viable cells, which are capable of responding to neuritogenic stimuli, without itself producing neurite outgrowth. The cell density had to be sufficient to promote cell viability but sparse enough to allow individual neurites to be delineated at the image analysis stage of the assay.
12. Pipeting heights and speeds need to be determined such that compound plates are thoroughly mixed and accurately pipeted to the cell plates. Compound addition to the cell plates, and subsequent mixing, needs to be performed very gently at a sufficient height within the well so that cell viability is not compromised. Cell plate lids were removed for as short a time as possible and sterility during the 48 h incubation was not found to be an issue.
13. The cell washer parameters need to be determined such that adequate washing was performed without disturbing the cells. Similarly, reagent addition needs to be performed gently to avoid disturbing the cells. Suboptimal parameters can sometimes be compensated for when imaging the plates, for example acquiring images from the edges of the wells if wash-off has occurred at the center. The secondary antibody solution is light sensitive and so was either added onto the PlateTrak deck just before it was required, and/or the whole PlateTrak protocol was run in subdued lighting. To reduce evaporation from the top plate in a stacker, and to allow antibody incubations to occur in the dark, an extra plate with blacked-out wells was added to each stacker but excluded from the number of plates entered into the software.
14. Antibody specificity and concentrations were determined in separate experiments. Antibodies which can be used at low concentrations are more suitable to this methodology, because of the relatively large volumes of reagents, which have to be prepared for automated liquid handling. Combining mono and polyclonal antibodies in the same assay allowed the effects of compounds on subpopulations of cells to be studied simultaneously, although the algorithm employed for analysis in this instance meant that each plate had to be read twice to achieve this.
15. The cell washer parameters need to be determined such that adequate washing is performed without disturbing the cells and the correct residual volumes are left in the wells. Pipeting heights and speeds also need to be determined for adequate transfer of reagents. Methanol's liquid property dictates that it required a substantial postaspirate air gap to avoid leakage during transfer. As the dye and secondary antibody solution are light sensitive, the assay was run in subdued lighting.
16. To save reagents in a small assay with only a few cell plates, the ×2 Fluo 3-AM dye and antibody solutions can be added from a 96-well microplate instead of a bird-feeder bottle and reservoirs. Pipeting heights would need to be changed accordingly.
17. Cell density need to be determined to allow adequate segmentation of the cells by the BD PathwayHT software and avoid clusters of cells being identified as one ROI.
18. Antibody concentrations were determined in separate experiments. In this assay, each well was only labeled for one cell type marker, thus three wells received the same compound treatment in the kinetic

experiment and then proceeded to be labeled with different antibodies. However, one could perform dual or triple labeling by using a combination of mono and polyclonal antibodies.

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