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# Enrichment of differentiated hNT neurons and subsequent analysis using flow-cytometry and xCELLigence sensing

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## HIGHLIGHTS

- New method to produce highly enriched cultures of differentiated hNT neurons.
- Additional methods to assess the viability and purity of the enriched neuronal cultures.
- xCELLigence measurement of the neuronal net adhesion.
- Method to analyse the cell surface phenotype of neuronal cells using flow cytometry.

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## ABSTRACT

*Background:* Human neurons (hNT neurons), obtained from the NTera2/D1 precursor cell line, are highly valued by many neuroscientists as isolation of adult human primary neuronal cells continues to elude us. hNT neurons are generated by differentiation of the NT2 precursors for a period of 4 weeks followed by 2 weeks of mitotic inhibition. This yields a heterogeneous population of neuronal phenotypes and underlying astrocyte precursors, the latter of which are very difficult to visualise using standard light microscopy. Such a mixed culture is acceptable for some applications (e.g. measurement of synaptic plasticity), whereas others (e.g. proteomics or transcriptomics) require almost pure cultures of hNT neurons.
*New method:* Here we describe a simple method for obtaining highly enriched cultures of hNT neurons following the first neuronal harvest and detail several additional methods, namely flow-cytometry and xCELLigence© biosensor technology, to rapidly and reliably determine the purity and viability of the cultures.
*Comparison with existing methods:* This method of enrichment for the neurons is novel and advances the end user applications of the cells.
*Results:* In addition, we apply the enrichment method to conduct analysis of cell-surface markers using flow-cytometry on the enriched neuronal cells. Furthermore, we apply this method to generate enriched neuronal cells on which we conduct analysis of cell-surface markers using flow-cytometry.
*Conclusions:* Collectively, this paper describes several new advances, which will create opportunities when using these cells and similar preparations, and provides the protocol for analysis of these cells using flow-cytometry and biosensor technology.



## 1. Introduction

Despite the advancements in biomedical research over the past 30 years, the isolation of adult human neuronal cells from brain tissue has remained elusive. Therefore, in order to conduct *in vitro* studies using neuronal cells, various neuronal model systems have been adopted. Options exist for primary neuronal cells, however, this can only be achieved from fetal brain tissue as the adult neuronal counterparts do not survive the isolation process. Thus, for

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adult human neuronal cells, there are only two options currently, those derived from stem-cells or from cell-lines. Of the latter, the NTera2/D1 cell-line can be differentiated to produce a heterogeneous culture of human neuronal-like cells, termed hNT neurons (Andrews, 1984; Pleasure et al., 1992). These neurons are a mixture of phenotypes capable of producing various neurotransmitters, which include dopamine, γ-aminobutyric acid (GABA) and acetylcholine (Zigova et al., 2000; Zeller and Strauss, 1995; Kondziolka et al., 2000; Nelson et al., 2002). In addition, hNT neurons have been shown to express glutamate receptors (Younkin et al., 1993; Hardy et al., 1994) and cannabinoid receptors (Goodfellow et al., 2011). hNT neurons have been shown to form functional synapses (Hartley et al., 1999) and are therefore a suitable model for the study of neurotransmission, synaptic plasticity and for neurotoxicology. hNT neurons have also been grafted into the rodent and human brain, with the latter clinical studies being conducted following ischemic stroke (Borlongan et al., 1998; Trojanowski et al., 1993; Kondziolka et al., 2000; Nelson et al., 2002). Collectively, these studies demonstrate the high value of the hNT neuronal model system to basic neuroscience and clinical applications.

Irrespective of the source or species of neurons used in studies, any neuronal cells obtained by differentiation of stem cells or precursors (as in the case of hNT neurons) will be prone to variations in the proportion of neurotransmitter phenotypes, or the purity of the differentiated cells. It is the purity of differentiated hNT cells that we address in this article. For some applications, such variation will not be an issue; in fact a mixed culture may even be required for some studies (Hartley et al., 1999). However, purity will become an issue for studies investigating the expression of neuronally expressed genes or proteins using proteomic/transcriptomic approaches, where the purity of the neuronal cells will be critically important. Purity also relates to functional studies where the objective is to measure direct neuronal signalling events or responses, without the confounding response from any stem cells or precursors.

NT2 precursors are known to produce astrocytes after a further period of differentiation, following removal of the neuronal cells (Bani-Yaghoub et al., 1999). Therefore, the astrocytic precursor cells are present at the time of the neuronal harvest. In our experience, it is very difficult to harvest the differentiated neuronal cells without some of the precursor cells being harvested too and the degree to which this occurs varies greatly between differentiations. Therefore, this method of neuronal harvest is a compromise between yield, viability and the number of neurons obtained. In our research, we typically require a high-yield of neuronal cells for downstream experiments. Therefore, our protocol relies on trypsinisation of the neurons to obtain the maximum yield; but this inevitably results in harvesting some of the astrocytic precursors. In contrast to the neuronal cells, the astrocytic precursors are very difficult to visualise with standard bright field microscopy, a factor not commonly acknowledged in the use of these cells. However, they can be identified on the basis of vimentin, βIII tubulin and MAP2 expression as well as by the size of their nuclei.

In this paper, we have developed a simple but effective method for reliably and consistently removing the majority of the astrocytic precursors from the neuronal harvest, thereby enriching the neuronal yield. This was achieved by adhesion of the precursors to plastic immediately following trypsinisation using xCELLigence biosensor guidance. Even following trypsinisation, the astrocytic precursors retain the ability to adhere rapidly (within an hour). This observation was first noted when we conducted xCELLigence biosensor experiments, where the mixed neuronal harvest produced a rapid and sizable increase in adhesion. In contrast, highly pure enriched neuronal cultures do not produce a strong xCELLigence signal, consistent with the weak adhesion properties of neurons. Subsequently we used flow-cytometry to assess the viability of the enriched neuronal cultures, and to confirm the loss of the pre-astrocytic cells. Finally, the enrichment method enabled a route to produce highly pure human neurons (hNT) that could be harvested without trypsin treatment and therefore facilitate assessment of cell-surface neuronal markers expressed by the hNT neurons using flow-cytometry.

## 2. Methods

### 2.1. Cell culture

The NTera2/D1 (NT2) cell line was purchased from the American Tissue Culture Collection (ATCC). Cells were cultured in DF10 (DMEM/F12 media supplemented with 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin–glutamine). Cells were split 1:4 when they reached 80% confluence. All cell culture reagents were purchased from Invitrogen (unless otherwise stated). Nunc tissue culture plastic was used throughout as the NT2 cell line has a strong preference for its properties over other plastics we have tested.

### 2.2. Differentiation of NTera2/D1 (NT2) cell line into neurons (hNT Neurons)

The protocol used to differentiate the NT2 cells was modified from the previously described cell aggregation methods of Paquet-Durand et al. (2003) and Jain et al. (2007) (Paquet-Durand et al., 2003; Jain et al., 2007). NT2 precursors were seeded at a density of $60 \times 10^6$ in 90 mm petri dishes (Greiner, GR664102). After 24 h, 10 μM retinoic acid (Sigma R2625) was added and following this cells were replated with fresh media and retinoic acid every 2–3 days for 2 weeks. Cells were then mechanically harvested and replated into T75 flasks (Nunc) with retinoic acid and media changes every 2–3 days for 9 days. At this point, hNT neurons were harvested using 0.05% trypsin-EDTA and seeded in a poly-D-lysine hydrobromide (10 μg/ml BD 354210) and Matrigel (BD 354234) coated flask in order to obtain the neuronal population. This flask along with the remaining cells were treated with mitotic inhibitors (1 mM uridine (Sigma U3003), 1 mM 5-Fluoro-2′-deoxyuridine (Sigma F0503) and 1 mM Cytosine β-D-arabinofuranoside (Sigma C1768)) in 5% FBS supplemented media for another week before hNT neurons from all flasks were harvested by trypsinisation for use. We referred to this harvest as the mixed culture as the harvest is a mixture of hNT neurons and astrocytic precursors (see Fig. 1). These cells were cultured on poly-D-lysine and Matrigel coated flasks with media (DF10) changes every 2–3 days.

### 2.3. Enrichment protocol of hNT neurons by selective removal of the astrocytic precursors

Flasks were trypsinised for 1–2 min in order to remove all neurons. Some of the underlying astrocytic-precursor cells are also removed during this step; this is unavoidable. At this stage, some of the harvest was used for xCELLigence adhesion, immunocytochemistry, or flow-cytometry experiments. The remainder of the harvested cells were seeded in 10 ml of 10% FBS supplemented DMEM/F12 media in uncoated Nunc T75 flasks and incubated for ∼1 h (the exact time-frame was determined by xCELLigence indication of cell adherence run in parallel). During this period, the astrocytic-precursor cells adhere rapidly, whereas the neuronal cells do not. After 1 h, the media (containing non-adherent cells; neurons) was removed carefully to prevent drawing off the non-neuronal cells. The harvested cells were counted and used for subsequent downstream applications at the same density as those obtained immediately following trypsinisation.

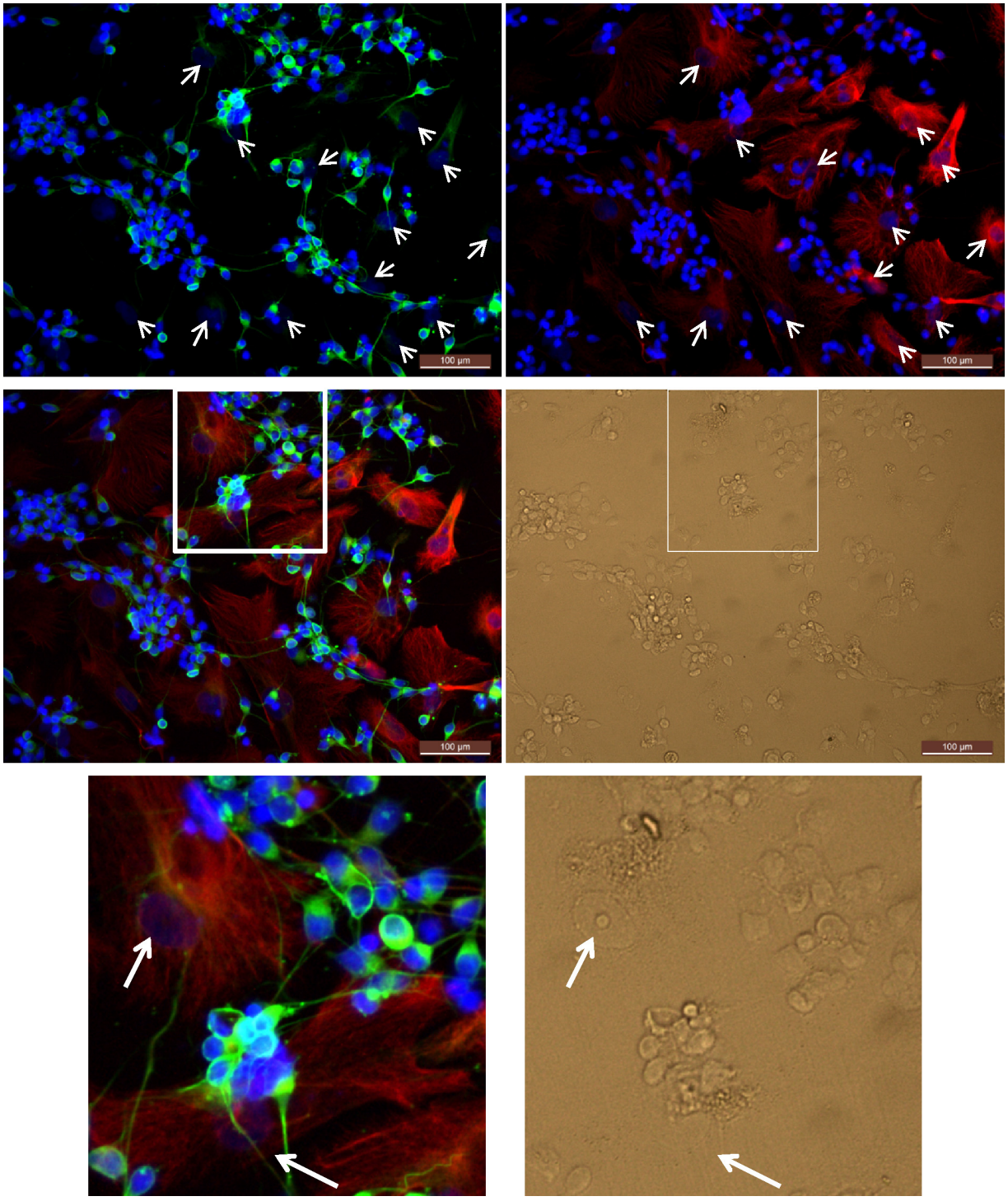


**Fig. 1.** Typical harvest of differentiated NT2 neurons is a mixture of neuronal cells and astrocyte-precursors. Seeding of the harvest at low density reveals the presence of cells with very large nuclei (white arrows in the fluorescent overlay and bright-field images). These large cells do not stain with βIII tubulin (green), which is expressed by the neuronal cells. The pre-astrocytic cells stain for vimentin (red) and have a diameter of 100–150 μm. These cells are very difficult to visualise in bright field (e.g. using a standard microscope) without staining. The white boxes in the low power images have been enlarged to highlight the size of the precursors and how difficult these are to see. Nuclei were stained with Hoechst. Scale bar represents 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 2.4. Immunocytochemistry

Cells were fixed 24 h (or as indicated in the legend) after seeding using 4% formalin for 10 min. Cells were then washed twice using PBST (PBS with 0.2% Triton X-100) without rocking in order to avoid loss of hNT neurons. Full details of all antibodies and conditions are summarised in Table 1. In brief, cells were incubated overnight with primary antibodies (anti-Vimentin (abcam ab15248) and anti-Beta-tubulin isotype III (Sigma T8660)) diluted in immunobuffer (PBS with 1% goat serum and 1% Triton-X-100). Cells were washed a further two times with PBST and incubated overnight with Alexafluor 488 anti-mouse IgG (total IgG) and AlexaFluor 594 anti-rabbit secondary antibodies (Molecular Probes # A-11012 and Molecular Probes # A-11001). Cells were

**Table 1**
Details of the specifications, sources, and titrations of each of the antibodies/stains used in this study. Note that the flow-cytometry antibodies are each directly conjugated with either FITC (FL1 channel) or APC (FL4 channel), where as the 7AAD is detected in the FL3 channel on the Accuri C6 flow cytometer (BD Biosciences).

| | Cat # | Host | Usage |
|---|---|---|---|
| **Antibody/stain** | | | |
| Vimentin | Abcam #15248 | Rabbit | 1:500 |
| βIII-tubulin | Sigma #T8660 | Mouse IgG2b | 1:500 |
| MAP-2 | Sigma #M4403 | Mouse $IgG_1$ | 1:100 |
| Nestin | Millipore #MAB5326 | Mouse IgG1 | 1:1000 |
| Anti-rabbit IgG Alexa 594 | Molecular Probes # A-11001 | Goat | 1:400 |
| Anti-mouse IgG Alexa 488 | Molecular Probes # A-11012 | Goat | 1:400 |
| Hoechst 33258 | Sigma #B1155 | NA | 1:500 |
| **Flow-cytometry antibodies** | | | |
| 7AAD (live-dead stain) | BD Biosciences #559925 | NA | |
| CD56 (NCAM) FITC conjugated | Biolegend #318304 | Mouse IgG1, κ | 1:40 |
| CD54 (ICAM-1) FITC conjugated | Biolegend #353107 | Mouse IgG1, κ | 1:40 |
| CD105 (Endoglin) FITC conjugated | Biolegend #323209 | Mouse IgG1, κ | 1:40 |
| CD138 (syndecan-1) APC conjugated | Biolegend #352307 | Mouse IgG1, κ | 1:40 |
| CD146 (MCAM) APC conjugated | Biolegend #342005 | Mouse IgG2a, κ | 1:40 |
| CD147 (neurothelin-1) APC conjugated | Biolegend #306209 | Mouse IgG1, κ | 1:40 |
| CD321 (JAM-1) FITC conjugated | Biolegend #353503 | Mouse IgG1, κ | 1:40 |

then incubated with Hoechst 33258 (Sigma B1155) for 20 min in PBST and then washed a further two times with PBST before imaging. Cells were imaged using the Leica DFC425 C microscope.

### 2.5. Flow cytometry

For the assessment of neuronal viability, mixed neuronal cultures and enriched neurons were harvested by trypsinisation and then counted. 100,000 cells were decanted into FACS tubes and stained for 15 min on ice with the cell viability stain 7AAD (Biolegend). 7AAD is only assimilated into dead or compromised cells. Following staining, cells were washed twice with FACS buffer (PBS containing 1% FBS) and spun at 300 × *g* for 5 min. Following the final decant, cells were reconstituted in 100 μl of FACS buffer and run on an Accuri C6 flow cytometer (BD Biosciences). Data were analysed using C6 software (Accuri; BD). For staining of cell surface proteins, enriched neuronal cells were harvested with Versene (non-enzymatic; trypsinisation destroys the cell surface targets detected by the live-stain antibodies). The purified neurons were counted and 50,000 cells were decanted into FACS tubes. Neurons were stained with a selection of directly conjugated monoclonal antibodies (Biolegend). All antibodies are listed in Table 1 Staining was conducted at 4 °C for 15 min followed by two washes with FACS buffer. Following the final spin at 300 × *g* (4 °C for 5 min), the supernatant was discarded and the cells reconstituted in 100 μl of FACs buffer. Samples were run immediately on an Accuri C6 flow-cytometer with fluidics set to slow.

All data were analysed using Accuri C6 software. Forward scatter (FSC) and side scatter (SSC) was used in conjunction with 7AAD staining to position the live (P1) and dead gates (P2). These gates were then used to selectively measure expression of the specific markers by the gated cells.

### 2.6. DP xCELLigence assays

The xCELLigence RTCA (Real Time Cell Analyser) system measures the level of cellular adhesion. As the cells adhere to the gold electrode array, which coats the bottom of the E-plates, the cells influence the electrical impedance of the array. This is converted to Cell Index by the xCELLigence software (version 21). The graphs are real-time generated outputs from the xCELLigence system. Each curve shows the mean (±SD) of 5 individual wells. The DP version of the xCELLigence system was used as described in Moodley et al. (2011) (Moodley et al., 2011) with some modifications. All wells were coated with poly-D-lysine and Matrigel prior to cell seeding in order to enhance neuronal adhesion. Following harvesting, as described above, cells were seeded immediately following trypsinisation and then again following the enrichment at a density of 5000 or 25000 cells per well in a total volume of 100 μl of DF10 as indicated in the figure legend. The xCELLigence assays were carried out in parallel with the immunocytochemistry and flow cytometry.

## 3. Results

### 3.1. Generation and validation of a typical harvest of differentiated human hNT neurons

Currently, the sources of adult human neuronal cells for *in vitro* studies are limited to those derived from stem-cells or differentiation of cell-lines. The hNT neurons represent one of the better characterised cell-line derived human neuronal cultures (Andrews, 1984; Hardy et al., 1994; Hartley et al., 1999; Jain et al., 2007; Megiorni et al., 2005; Pleasure et al., 1992; Saporta et al., 2000; Schwartz et al., 2005; Younkin et al., 1993; Zigova et al., 2000; Lee and Andrews, 1986; Goodfellow et al., 2011). The hNT neurons are obtained by differentiation of NTera2/D1 precursor cells. Multiple different differentiation protocols exist, with most producing human neuronal cells positive for βIII tubulin and MAP-2, and negative for nestin (stem cells), vimentin and GFAP (astrocytes) within about 6–7 weeks of differentiation with retinoic acid and mitotic inhibitors.

Fig. 1 depicts a typical hNT neuronal harvest in which the βIII tubulin-positive neuronal cells are clearly evident. At high magnification, the finer neuronal dendritic processes are usually visible. Even though the neuronal cells are clearly abundant (Fig. 1), there are also cells negative for βIII tubulin but positive for vimentin, present in the neuronal harvest. These large vimentin positive cells are precursors to astrocytes, which can be further differentiated into astrocytes (Bani-Yaghoub et al., 1999). These precursors are much larger in size, typically 150–200 μm in diameter and stain positively for the intermediate filament protein vimentin. They are however, very difficult to see using standard bright-field microscopy. It is therefore quite easy to underestimate how many astrocytic-precursors are actually present in the neuronal harvest simply by bright-field analysis. As well as being able to identify the neurons and pre-astrocytic cells by their specific cell markers, nuclei size is an indicator of cell type. The precursor cells have large nuclei typically >25 μm, whereas the neuronal nuclei are much smaller (~5 μm) and stain more intensely with Hoechst. In our experience, the purity of the initial neuronal harvest is variable,

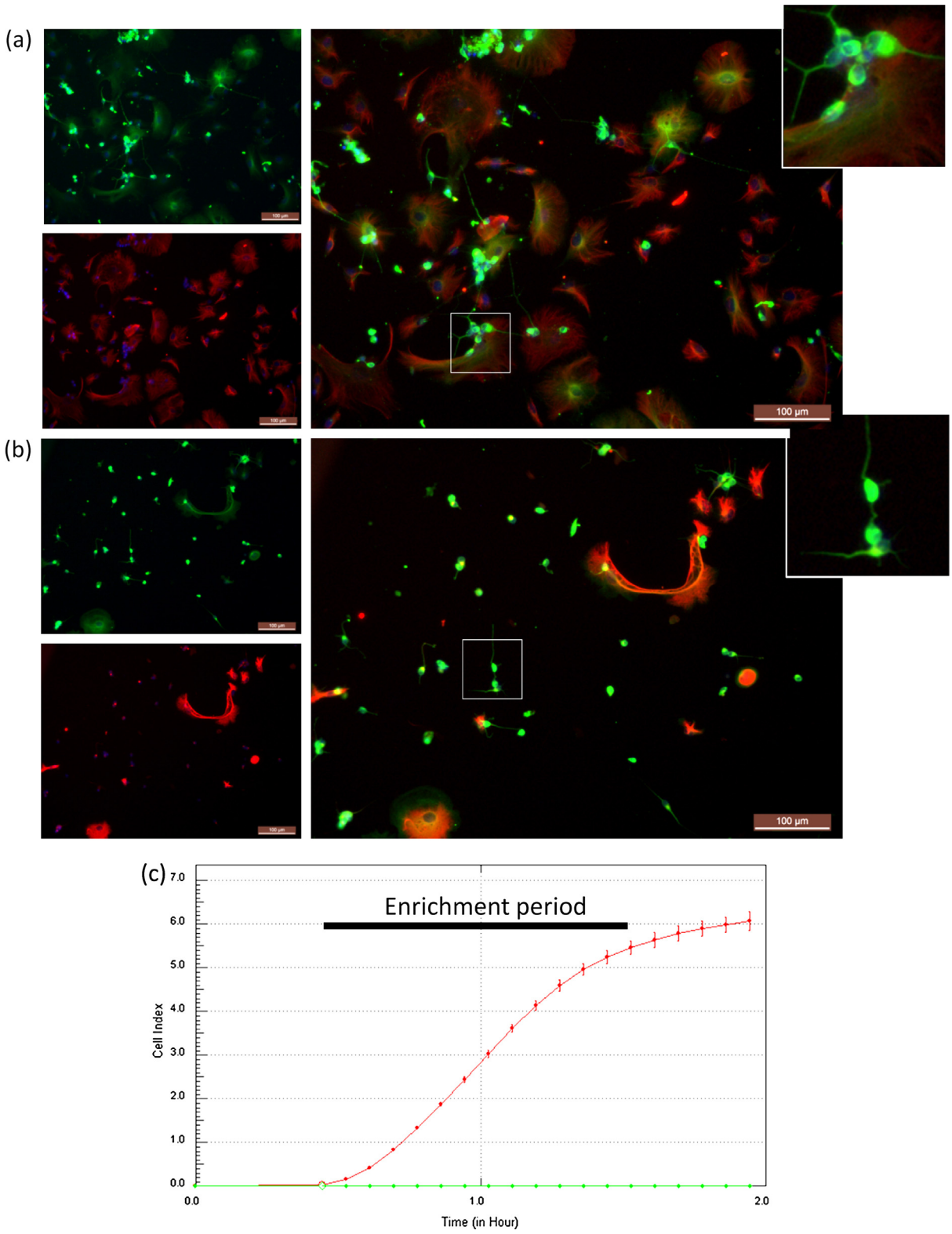


**Fig. 2.** Comparison of mixed neuronal cultures and enriched hNT neuronal cultures. The NT2 precursors differentiate into neuronal cells and astrocyte-like cells after 6 week and 10 week differentiation protocols, respectively. The neuronal cells are harvested off a bed of immature-astrocyte like cells. Typically, the first neuronal harvest is a mixture of the neurons and underlying immature astrocytes. (a) Shows the mixture of βIII-tubulin (green) positive neurons, which are vimentin negative and the numerous immature astrocyte-like cells (vimentin positive; red), typical of the first neuronal harvest. (b) Shows the neuronal culture greatly enriched for neurons, by removal of the immature astrocyte like cells. The enrichment method we have developed typically produces neuronal cultures that are 85-95% pure neurons. Nuclei were counter-stained with Hoechst (blue) following immunocytochemistry for βIII-tubulin (green) and vimentin (red). (c) xCELLigence biosensor data showing adhesion of the mixed culture (red curve), outlining the period used during the enrichment protocol. Media only measurement is the green line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

but routinely at least 20% are astrocytic precursors. This represents a significant amount of cellular-mass especially for down stream analysis of proteins, gene expression or functional responses.

The images in Fig. 1 were acquired after 2–3 days in culture when the neuronal cells are still relatively singular, however, the neuronal cells tend to clump together the longer they are left in culture. In cultures left for several weeks, the neuronal cells form massive spheres of neurons containing hundreds of cells as shown in Supplemental Fig. 1

### 3.2. Enrichment of differentiated human hNT neurons

For certain applications (e.g. methods involving transcriptomics, or proteomics), the use of highly pure or enriched neuronal cultures is desirable. We have modified our hNT differentiation protocol (Raos et al., 2013; Unsworth et al., 2011), to better facilitate enrichment of the neuronal yield. This adapted method preferentially selects the immature-astrocyte like cells by rapid adhesion to plastic (the pre-astrocytic cells have a strong preference for Nunc), and leaves the majority of the neurons in suspension. This simple, but effective method was conceptualised following earlier observations revealing how rapidly the astrocyte-like cells adhere using xCELLigence biosensor technology and that they do so much faster than the neurons.

Fig. 2 shows representative images of the same hNT neuronal harvest before enrichment (Fig. 2a) and after enrichment (Fig. 2b). During the enrichment protocol, most of the precursor cells are removed as can be seen in Fig. 2b. The enrichment period was determined using xCELLigence biosensor technology to monitor the adhesion of the cells in real-time (Fig. 2c). Although the absolute extent of adhesion varied slightly between experiments (as expected) the neurons were typically collected after 1 h. This coincided with the plateau of the Cell Index curve, indicative that the initial adhesion phase was complete. Biosensor technology (xCELLigence) is very powerful for revealing these events. Longer periods of enrichment are certainly possible, but in our experience this occurs at the cost of neuronal yield, as longer enrichment periods facilitate adhesion of the neurons too. Thus, there is a compromise between absolute purity and yield. Ultimately, the duration of this period should be empirically determined using biosensor technology, analogous to our use of the xCELLigence system.

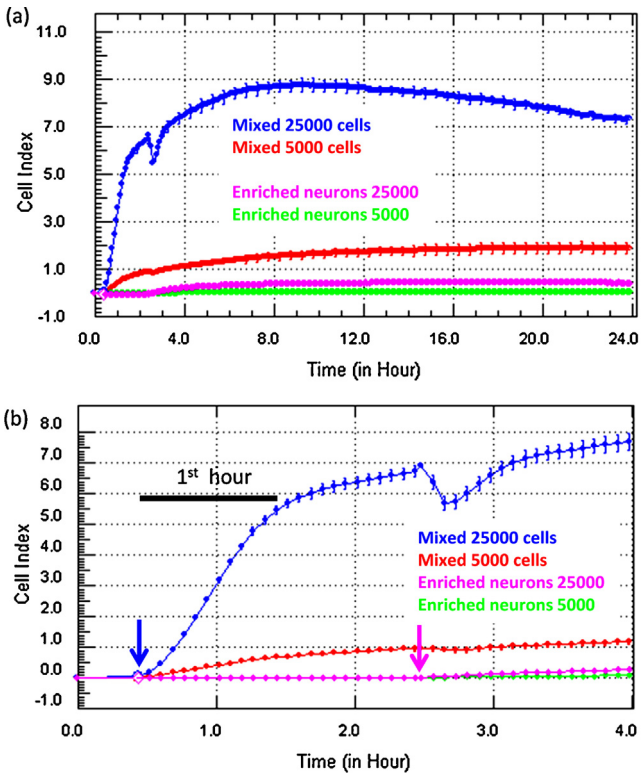


**Fig. 3.** xCELLigence data showing Cell Index curves from mixed and subsequently enriched hNT neuronal cultures. xCELLigence biosensor technology was used to measure the net adhesion of the mixed neuronal culture and the enriched neurons. Panel (a) shows the adhesion measurement across the initial 24 h period, with (b) highlighting the first few hours encompassing the enrichment period. Following the first neuronal harvest, the mixed neuronal culture was seeded into xCELLigence E16 plates at 25,000 (blue curve) and 5000 (red curve) cells per well. The blue arrow demarcates when the mixed culture was seeded (b). Then the enriched neuronal culture was produced (see methods). The subsequently enriched neuronal cells were harvested, counted and seeded at 25,000 cells (pink curve) and 5000 cells (green curve); time of addition highlighted by the pink arrow. The high Cell Index values were only observed with the mixed neuronal cultures containing the immature-astrocyte like cells. Data show the mean ±SD ($n = 5$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

### 3.3. Analysis of purity of human hNT neuron harvests using xCELLigence biosensor technology

Fig. 3 shows the Cell Index adhesion curves of mixed neuronal cultures and neuronal cells following the enrichment method. The top panel (a) shows the initial 24 h period following seeding of the cells. This time-frame reveals that the rate of adhesion in the mixed culture is rapid and reaches an impressive Cell Index of ~90 within 8 h. We would consider this very strong, in terms of cellular adhesion. However, it was found that post enrichment the maximum Cell Index does not get above 10. This proved to be a very useful observation, showing that upon removal of the astrocytic precursors, the highly enriched neuronal cells do not produce a high Cell Index. Thus, their net cellular adhesion is low even at a high density of 25,000 cells per well. Panel (b) focuses on the initial 4-h adhesion period, which shows the increase in Cell Index following addition of the mixed neuronal culture. The blue curve represents 25,000 cells and the red is 5000 cells, of the mixed neuronal culture. The Cell Index values obtained for the 25,000 cells are approximately 5- to 6-fold higher than for the 5000 cells. Note that the adhesion is greatest within the first hour after seeding. This period represents the enrichment period.

This technology provides us with a new method to assess the presence of astrocytic precursors in the neuronal harvest and thus indicates the purity of the neuronal cultures. Furthermore, while the Cell Index of the enriched neuronal population never rises above one it is still sensitive enough to detect the 5-fold difference between the two different cell densities of the enriched neuronal cells. The demonstration of this level of sensitivity gives further validation for the use of this platform in this and other applications.

### 3.4. Analysis of hNT neuronal viability and purity using flow-cytometry

The assessment of viability following harvesting of the neuronal cells can be done using flow-cytometry. The cell exclusion dye 7AAD is only taken up through compromised cell membranes. Thus along with light scatter (forward and side) is used to define the live and dead neuronal populations. This is very important given the particular sensitivity of neuronal cells to the harvesting process. Fig. 4 shows four panels revealing the cellular region of interest (R1) based on forward/side scatter. The area outside of R1 contains debris, which originates from the Matrigel ECM coating and cellular debris from compromised cells. Events outside of R1 are excluded from further analysis. R1 contains the Live-gate (P1) which is 7AAD negative and the Dead-gate (P2), which is 7AAD positive. P1 also represents the single homogeneous population of neuronal cells, but avoids potential doublets

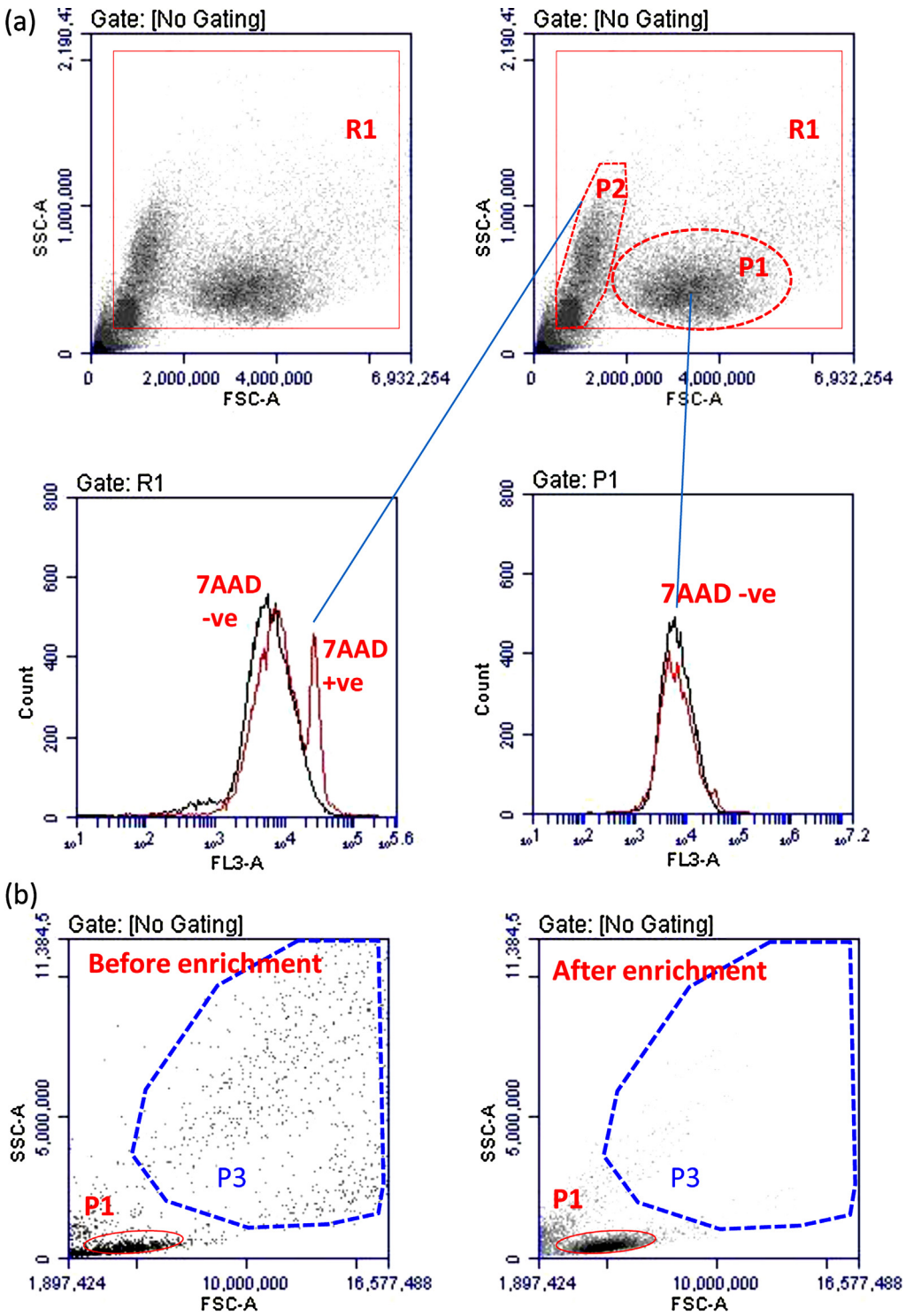


**Fig. 4.** Assessment of neuronal viability and enrichment using flow cytometry. (a) Discrimination of the viable and compromised hNT neurons based on 7AAD staining and forward/side scatter. In the dot plots, the region containing the cells is designated as R1. Within R1, the live-gate is designated as P1 and the 7AAD positive gate is P2. In the overlay plots, the 7AAD signal is in red, whereas the neuronal auto fluorescence is black. The rightward shift for 7AAD (red), represents compromised cells. The magnitude of the rightward shifted peak gives the extent of neuronal compromise. (b) Comparison of forward and side scatter plots for the mixed neuronal harvest and following enrichment. The astrocytic-precursor cells have a much larger forward/side scatter (P3) and substantially fewer remain following enrichment. In contrast, more cells are clearly present in the P1 gate following enrichment. Each plot in (b) shows the same number of cells, with each dot representing a single cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

or the larger astrocytic precursor cells. This approach is standard in terms of defining the Live-gate (P1), which was used in the subsequent cell surface expression analysis. The proportion of neuronal cells positive for 7AAD ranged from 18% to 25% between preparations.

In addition to showing the viability of the neuronal population using this method, we were also able to demonstrate the removal of the larger pre-astrocytic population. Fig. 4b illustrates the almost complete removal of the cells in the P3 region. These cells have a large forward and side scatter, consistent with being the large

pre-astrocytic cells. Thus, this method provides a rapid and reliable indication that the pre-astrocytic cells have been removed and of the viability of the resultant enriched neuronal cells.

### 3.5. Use of enriched neuronal cultures for analysis of cell surface phenotype by flow-cytometry

Flow-cytometry is a very powerful tool for analysis of multiple markers simultaneously. However, the use of flow-cytometry in neuroscience is quite limited. This is probably because effective use of flow-cytometry for phenotypic analysis is reliant on two major factors; the first of which is access to primary antibodies directly conjugated with fluorescent tags (e.g. FITC/488, PE, and APC). These antibody preparations are somewhat lacking for neuroscience applications, even though they are commonly and widely used in immunology. The second factor, which is more limiting, is having a renewable source of viable single neurons, prepared without trypsinisation (or other enzyme based methods). Trypsin provides good single cell suspensions but destroys the epitopes bound by the flow-antibodies. Here our method of enrichment yields highly purified neuronal cells that can be harvested using Versene to provide single cell suspensions, thus avoiding Trypsin.

The expression of various cell surface adhesion molecules was assessed using flow-cytometry (Fig. 5). These included markers known to be expressed within the brain by neurons (CD56 and CD147). The hNT neurons express CD56 (NCAM-1; positive control stain), CD138, CD146 and CD147 As expected they completely lacked expression of the endothelial cell adhesion molecules CD105 and CD325 In addition, they were negative for the adhesion molecules CD54 (ICAM-1) (Fig. 5), CD102 (ICAM-2) and CD50 (ICAM-3) (data not shown), which are involved in the interaction with immune cells. This is the proof-of-concept of using the highly enriched neuronal cells for flow-cytometry analysis of cell surface markers. This method could be used to investigate expression of any cell surface target (neurotransmitter receptors, channels and other adhesion molecules) expressed by neuronal cells or sub-populations of neurons, contingent on availability of suitable antibodies.

This confirmation of expression of neuronal specific cell surface markers as well as the lack of endothelial cell adhesion molecules provides a robust method to further validate not only the purity of the culture but also the use of this cell line as a model for human neurons.

## 4. Discussion

The use of hNT neurons as an *in vitro* model of human neurons is widespread, and extensive characterisation of these cells has been carried out. However, as these cells are generated by differentiation from the precursor cell line NTera2/D1, there is variability between differentiations in regards to the purity of the neuronal harvest. In this investigation, we demonstrated the high percentage of astrocytic-precursor cells that are typically present in an hNT neuronal harvest, which in our opinion is not commonly acknowledged in the use of these cells. The extent of this impurity was demonstrated not only using immunocytochemistry, the method used in most studies, but more accurately using both flow cytometry and the xCELLigence biosensor platform. While this impurity of harvest may be of little significance for some studies, in other applications such as transcriptomics, proteomics and indeed in potential transplantation of these cells into the human brain, a pure neuronal population is essential for obtaining accurate and reliable results.

There have been numerous methods published describing variations on the differentiation protocol for hNT neurons (Andrews, 1984; Jain et al., 2007; Paquet-Durand et al., 2003; Pleasure et al., 1992) which may increase the yield of cells or decrease the time to harvest. Here we have provided an extension to these methods, which effectively eliminates the large majority of non-neuronal cells thereby leaving a highly pure culture of hNT neurons. We have also validated this method using flow cytometry and the xCELLigence biosensor platform to show the removal of the larger pre-astrocytic population and the viability of the neurons after the replate step.

In addition to the aforementioned applications in which a pure neuronal culture would be advantageous, it is also essential for other applications. In our lab the hNT neurons are being utilised for cell patterning on parylene coated silicon chips (Unsworth et al., 2010), with the goal of achieving single cell patterning on an electrode microarray, a challenge which requires a highly pure neuronal population. In addition, and as expanded upon below, in order to analyse these hNT neurons using flow cytometry, a neuronal population that can be harvested without the use of trypsin is required and can be achieved using this technique. Finally, in order to be sure that results obtained using the xCELLigence biosensor platform are indeed due to the hNT neurons and not astrocytes or their precursors, a highly pure culture is required. While there are some studies in which the xCELLigence biosensor platform has been used to analyze the HT22 cell line, murine neuronal cells (Diemert et al., 2012; Grohm et al., 2010) and SH-SY5Y neurons (Dwane et al., 2013), to our knowledge, this is the first study in which the hNT neurons have been analysed using this technology. While it is possible to detect the hNT neurons even after enrichment using this platform, the cell index is extremely low in comparison to other cell lines we have experienced. In order to detect effects upon these cells, a very high density of seeding would be required, a limitation when these cells are generated using a lengthy protocol. Furthermore, those using the hNT neurons with xCELLigence should be aware that high cell indexes are likely due to non-neuronal cells within the culture. This effect may also extend to neuronal cells of primary origin or from stem cells. Thus, the presence of other non-neuronal cells should be considered. The fact that the difference in cell number even after enrichment could be detected using this technology is further validation for its sensitivity and use in future studies. The small but detectable Cell Index from the hNT neurons is consistent with the recent report from Kiely and colleagues (Dwane et al., 2013) who observed similar values form SH-SY5Y neurons. The Cell Index values observed for the pre-astrocytic cells are comparable to the high Cell Index values (8–12) from NT2 derived astrocytes we have reported previously (Van kralingen et al., 2013).

This enrichment technique also provides further opportunity to characterize the hNT neurons, by the use of flow cytometry. By obtaining a highly pure population that can be harvested without the use of trypsin, the cell surface phenotype of the neuronal culture is preserved allowing it to be further characterized. While we already know that the hNT neurons are a heterogeneous population (Saporta et al., 2000), the use of flow cytometry will allow us to identify specific phenotypes and their prevalence within a population of neurons. The ability to do this may be useful in selecting cells for transplantation as part of a potential treatment for neurological disease, an exciting prospect which is already being investigated by several groups (Nelson et al., 2002; Garbuzova-Davis et al., 2002; Hurlbert et al., 1999; Borlongan et al., 1998; Kondziolka et al., 2000). Furthermore, by being able to eliminate the astrocytes/precursor cells there is potential to identify cell surface markers, which are altered by the presence or absence of these other cell types. By being able to better decipher the phenotype of the hNT neuronal population we can potentially also further determine/validate the usefulness of these cells as a model for human neurons.

While this is a potentially useful technique, the main limitation to this method of enriching is the significant loss of neurons. The longer the replate step, the greater the elimination of non-neuronal

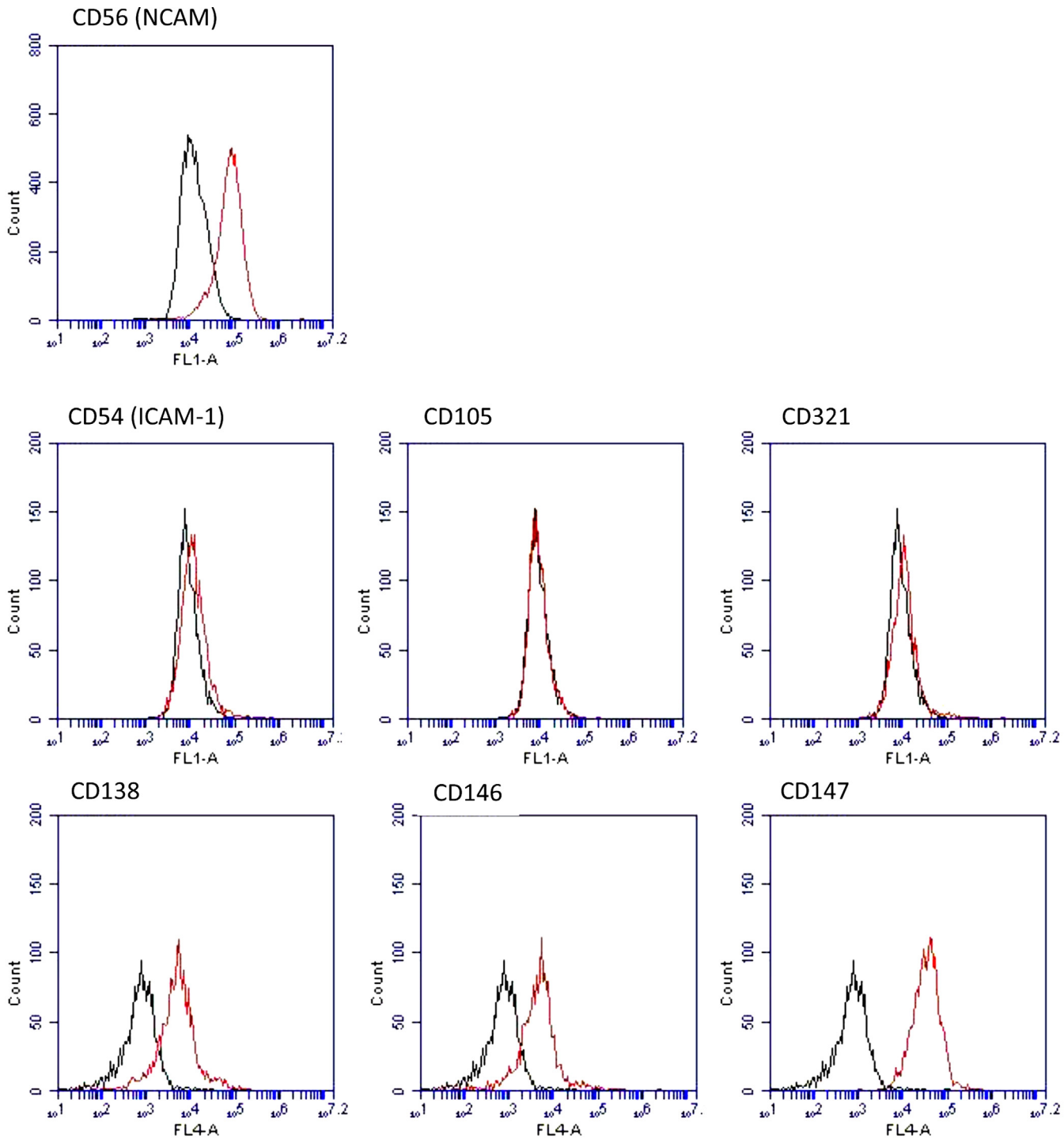


**Fig. 5.** Neuronal enrichment protocol facilitates analysis of cell-surface phenotype of enriched hNT neuronal cells using flow-cytometry. Neuronal cells positioned in the neuronal live gate (excludes all 7AAD positive cells) were included for analysis. hNT neuronal cells completely lack classical markers expressed by human vascular endothelial cells including CD105 and CD321 (JAM-1), and have negligible expression of ICAM-1 (CD54). In contrast, hNT neurons are positive for various matrix adhesion molecules expressed throughout the human CNS including CD138 (syndecan-1), CD146 (MUC-18; MCAM) and CD147 (Neurothelin-1). On the flow-histograms, the specific staining from the CD antibody is shown in red, whereas the unstained auto fluorescence is the black curve. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

cells, however, more neuronal cells are also lost as they begin to adhere to the underlying cells. For this reason, this technique would be specifically used for applications in which an extremely pure culture is essential. In addition, while a pure neuronal population is required for some applications, the presence of the pre-astrocytic cells is likely a more physiologically relevant model as this mixed culture is more representative of the neurons and glial cells in the brain. The importance of the interaction of these two cell types was demonstrated by Hartley et al. (1999) in which it was shown that the underlying NT2 astrocytes were required in order to detect functional synapses in the neurons. This study demonstrates the value of having the ability to remove the non-neuronal cells or leave them in the culture, thereby revealing their significance and effect upon the neuronal cells.

This study has provided an extension to a number of previously published methods, which enables the acquisition of a highly enriched culture of hNT neurons, which can be harvested without the use of trypsin due to the almost complete elimination of the underlying pre-astrocytic cells. Moreover, we demonstrate the value of biosensor technology (xCELLigence) and flow cytometry for studying the hNT neuronal cells.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jneumeth.2014.02.004.

## References

Andrews PW. Retinoic acid induces neuronal differentiation of a cloned human embryonal carcinoma cell line in vitro. Developmental Biology 1984;103:285–93.

Bani-Yaghoub M, Felker JM, Naus CC. Human NT2/D1 cells differentiate into functional astrocytes. Neuroreport 1999;10:3843–6.

Borlongan CV, Tajima Y, Trojanowski JQ, Lee VMY, Sanberg PR. Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N Cells) promotes functional recovery in ischemic rats. Experimental Neurology 1998;149:310–21.

Diemert S, Dolga AM, Tobaben S, Grohm J, Pfeifer S, Oexler E, et al. Impedance measurement for real time detection of neuronal cell death. Journal of Neuroscience Methods 2012;203:69–77.

Dwane S, Durack E, Kiely PA. Optimising parameters for the differentiation of SH-SY5Y cells to study cell adhesion and cell migration. BMC Research Notes 2013;6:366.

Garbuzova-Davis S, Willing AE, Milliken M, Saporta S, Zigova T, Cahill DW, et al. Positive effect of transplantation of hNT neurons (NTera 2/D1 Cell-Line) in a model of familial amyotrophic lateral sclerosis. Experimental Neurology 2002;174:169–80.

Goodfellow CE, Graham SE, Dragunow M, Glass M. Characterization of NTera2/D1 cells as a model system for the investigation of cannabinoid function in human neurons and astrocytes. Journal of Neuroscience Research 2011;89:1685–97.

Grohm J, Plesnila N, Culmsee C. Bid mediates fission, membrane permeabilization and peri-nuclear accumulation of mitochondria as a prerequisite for oxidative neuronal cell death. Brain, Behavior, and Immunity 2010;24:831–8.

Hardy M, Younkin D, Tang C-M, Pleasure J, Shi Q-Y, Williams M, et al. Expression of non-NMDA glutamate receptor channel genes by clonal human neurons. Journal of Neurochemistry 1994;63:482–9.

Hartley RS, Margulis M, Fishman PS, Lee VMY, Tang C-M. Functional synapses are formed between human NTera2 (NT2N, hNT) neurons grown on astrocytes. Journal of Comparative Neurology 1999;407:1–10.

Hurlbert MS, Gianani RI, Hutt C, Freed CR, Kaddis FG. Neural transplantation of hNT neurons for Huntington's disease. Cell Transplantation 1999;8:143–51.

Jain P, Cerone MA, Leblanc AC, Autexier C. Telomerase and neuronal marker status of differentiated NT2 and SK-N-SH human neuronal cells and primary human neurons. Journal of Neuroscience Research 2007;85:83–9.

Kondziolka D, Wechsler L, Goldstein S, Meltzer C, Thulborn KR, Gebel J, et al. Transplantation of cultured human neuronal cells for patients with stroke. Neurology 2000;55:565–9.

Lee VM, Andrews PW. Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. Journal of Neuroscience: The Official Journal of the Society for Neuroscience 1986;6:514–21.

Megiorni F, Mora B, Indovina P, Mazzilli MC. Expression of neuronal markers during NTera2/cloneD1 differentiation by cell aggregation method. Neuroscience Letters 2005;373:105–9.

Moodley K, Angel CE, Glass M, Graham ES. Real-time profiling of NK cell killing of human astrocytes using xCELLigence technology. Journal of Neuroscience Methods 2011;200:173–80.

Nelson PT, Kondziolka D, Wechsler L, Goldstein S, Gebel J, Decesare S, et al. Clonal human (hNT) neuron grafts for stroke therapy: neuropathology in a patient 27 months after implantation. American Journal of Pathology 2002;160:1201–6.

Paquet-Durand F, Tan S, Bicker G. Turning teratocarcinoma cells into neurons: rapid differentiation of NT-2 cells in floating spheres. Developmental Brain Research 2003;142:161–7.

Pleasure SJ, Page C, Lee VM. Pure, postmitotic, polarized human neurons derived from NTera 2 cells provide a system for expressing exogenous proteins in terminally differentiated neurons. Journal of Neuroscience: The Official Journal of the Society for Neuroscience 1992;12:1802–15.

Raos BJ, Unsworth CP, Costa JL, Rohde CA, Doyle CS, Bunting AS, et al. Infra-red laser ablative micromachining of parylene-C on $SiO_2$ substrates for rapid prototyping, high yield, human neuronal cell patterning. Biofabrication 2013;5:025006.

Saporta S, Willing AE, Colina LO, Zigova T, Milliken M, Daadi MM, et al. In vitro and in vivo characterization of hNT neuron neurotransmitter phenotypes. Brain Research Bulletin 2000;53:263–8.

Schwartz CM, Spivak CE, Baker SC, Mcdaniel TK, Loring JF, Nguyen C, et al. NTera2: a model system to study dopaminergic differentiation of human embryonic stem cells. Stem Cells and Development 2005;14:517–34.

Trojanowski JQ, Mantione JR, Lee JH, Seid DP, You T, Inge LJ, et al. Neurons derived from a human teratocarcinoma cell line establish molecular and structural polarity following transplantation into the rodent brain. Experimental Neurology 1993;122:283–94.

Unsworth CP, Graham ES, Delivopoulos E, Dragunow M, Murray AF. First human hNT neurons patterned on parylene-C/silicon dioxide substrates: combining an accessible cell line and robust patterning technology for the study of the pathological adult human brain. Journal of Neuroscience Methods 2010;194:154–7.

Unsworth CP, Holloway H, Delivopoulos E, Murray AF, Simpson MC, Dickinson ME, et al. Patterning and detailed study of human hNT astrocytes on parylene-C/silicon dioxide substrates to the single cell level. Biomaterials 2011;32:6541–50.

Van kralingen C, Kho DT, Costa J, Angel CE, Graham ES. Exposure to inflammatory cytokines IL-1beta and TNFalpha induces compromise and death of astrocytes; implications for chronic neuroinflammation. PLoS ONE 2013;8:e84269.

Younkin DP, Tang CM, Hardy M, Reddy UR, Shi QY, Pleasure SJ, et al. Inducible expression of neuronal glutamate receptor channels in the NT2 human cell line. Proceedings of the National Academy of Sciences of the United States of America 1993;90:2174–8.

Zeller M, Strauss WL. Retinoic acid induces cholinergic differentiation of NTera 2 human embryonal carcinoma cells. International Journal of Developmental Neuroscience 1995;13:437–45.

Zigova T, Barroso LF, Willing AE, Saporta S, Mcgrogan MP, Freeman TB, et al. Dopaminergic phenotype of hNT cells in vitro. Developmental Brain Research 2000;122:87–90.