

# Defining cell populations with single-cell gene expression profiling: correlations and identification of astrocyte subpopulations

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## ABSTRACT

**Single-cell gene expression levels show substantial variations among cells in seemingly homogenous populations. Astrocytes perform many control and regulatory functions in the central nervous system. In contrast to neurons, we have limited knowledge about functional diversity of astrocytes and its molecular basis. To study astrocyte heterogeneity and stem/progenitor cell properties of astrocytes, we used single-cell gene expression profiling in primary mouse astrocytes and dissociated mouse neurosphere cells. The transcript number variability for astrocytes showed lognormal features and revealed that cells in primary cultures to a large extent co-express markers of astrocytes and neural stem/progenitor cells. We show how subpopulations of cells can be identified at single-cell level using unsupervised algorithms and that gene correlations can be used to identify differences in activity of important transcriptional pathways. We identified two subpopulations of astrocytes with distinct gene expression profiles. One had an expression profile very similar to that of neurosphere cells, whereas the other showed characteristics of activated astrocytes *in vivo*.**

## INTRODUCTION

Brain contains three neuroectoderm-derived cell types: astrocytes, neurons and oligodendrocytes. They all originate from the same multipotent neural stem cells. Traditionally, astrocytes were viewed as a homogeneous cell population that predominantly supports neuronal functions. Recent findings point to many additional functions of astrocytes in health and disease, including control of the number and the function of neuronal synapses (1).

Cell diversity is commonly studied with immunohistochemical analysis and gene expression profiling. Both methods have several limitations. Immunohistochemical and immunocytochemical analyses are restricted to few markers and cannot be used in a truly quantitative manner. Cell types are often defined by the presence or absence of specific markers. Such binary approach to define cell types or functional states is coarse and thus not suitable to detect subpopulations differing only in the degree of expression by individual genes. For example, the hallmark of activated astrocytes is the upregulation of the intermediate filament proteins glial fibrillary acidic protein (*GFAP*), vimentin (*Vim*) and nestin (*Nes*) (2). Gene expression profiling can in principle be applied on the whole transcriptome. Such measurements are in general limited to large cell populations and thus only reflect global transcript levels. Consequently, any important heterogeneity among the cells remains undetected.

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With single-cell gene expression profiling we can study heterogeneity among and within cell types in a precise manner. The main obstacle to single-cell measurements has been the absence of sensitive and reproducible methods to measure small numbers of molecules. Single-cells can be collected by microaspiration, flow cytometry and laser capture microdissection (3–8). Transcript levels are then measured using microarrays or reverse transcription quantitative real-time PCR (RT-qPCR). Microarray measurements require a pre-amplification step (9,10), while RT-qPCR has the sensitivity to detect a single mRNA molecule. However, pre-amplification is also needed for RT-qPCR if many transcripts are to be quantified. To characterize well-defined cell types, cells can be enriched/selected for using specific antibodies. Antibody based enrichment is compatible with all cell collection methods, while morphology can only be used as a selection criterion when collecting cells with laser capture microdissection and microaspiration from tissues. Single-cell analysis is refining cell type characterization (11–13). Most single-cell studies so far have relied on preexisting knowledge about the analyzed cells. For instance, hematopoietic subpopulations can be isolated by flow cytometry using well-established surface markers (3,14). Specific types of neurons can be collected based on localization and/or immunohistochemistry using laser capture microdissection or microaspiration (4–7). Single-cell gene expression profiling can also be used to identify new subpopulations of cells from heterogeneous cell populations. This approach is still largely unexplored and tools for identification and classification of subpopulations are missing. Furthermore, transcription takes place in bursts in mammalian cells (15,16). Consequently, mRNA levels are highly variable even within a homogeneous cell population. Thus, gene expression levels between cells cannot be analyzed in the same way as in conventional cell population studies.

In this study, we have developed a strategy to identify and characterize subpopulations of cells. We show how subpopulations of primary astrocytes can be identified and defined by differences in correlated expression levels rather than by binary on/off responses from selected genes. Further, we show how transcriptional correlations can be used to reveal biologically important interactions between genes at a cellular level. Based on this platform, we identified two subpopulations of astrocytes, one with features commonly ascribed to activated astrocytes *in vivo* and one astrocyte subpopulation sharing characteristics with neurosphere cells.

## MATERIALS AND METHODS

### Animals and cell cultures

Primary astrocyte and neurosphere cultures were generated from mouse brains. The mice were housed in standard cages in a barrier animal facility with a 12-h light/dark cycle and feed *ad libitum*. All experiments were conducted according to protocols approved by the Ethics Committee of the University of Gothenburg.

Primary astrocytes were prepared from post-natal day (P) 1 mouse brains and cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml of penicillin and 0.1 mg/ml streptomycin (all Invitrogen) as described (17). After 10–11 days *in vitro*, almost confluent astrocyte cultures were harvested for gene expression profiling.

Neurosphere cultures were generated from P4 brains with cerebellum removed. These were dissected in Leibovitz medium (Invitrogen) and digested enzymatically [0.1% trypsin, 0.5 mM EDTA in Hank's balanced salt solution; (Sigma-Aldrich)] and mechanically dissociated into a single-cell suspension. Cells ( $\sim 10^5$ ) were cultured in Neurobasal medium (Invitrogen) containing 2 mM L-glutamine, 100 U/ml of penicillin, 0.1 mg/ml streptomycin, 1X B27, 20 ng/ml basic FGF (all Invitrogen), 20 ng/ml EGF (Stemcell Technologies), 1 U/ml heparin (Sigma-Aldrich) and 0.25  $\mu$ g/ml Fungizone (Bristol-Meyers Squibb). After 9 days *in vitro* the cells were used for gene expression profiling.

For cell population measurements, mice were killed at P1, P4 and P60. Whole brains were dissected (P4 with cerebellum removed) and stored at  $-80^\circ\text{C}$ . Total RNA was extracted using RNeasy Lipid Tissue Mini Kit, including DNase treatment (Qiagen).

### Single-cell isolation and cDNA synthesis

Astrocytes were washed twice in PBS and treated with 0.25% Trypsin/EDTA (Invitrogen) for 2 min to dissociate cells. Single-cells were kept in either PBS supplemented with 2.5% FCS or in astrocyte culture medium and kept on ice. The difference in cell medium had a negligible effect, so the astrocyte data were pooled for analysis. Neurospheres were enzymatically dissociated into single-cell suspensions with TrypLE (Invitrogen) and kept in neurosphere medium on ice until cell sorting. Cell aggregates were removed by filtering with 40  $\mu$ m cell strainer (Becton Dickinson). Single cells were sorted with a BD FACSAria (Becton Dickinson) into 96-well plates (Sarstedt) containing 5  $\mu$ l mQ water per well. Samples were frozen at  $-80^\circ\text{C}$  until subsequent analysis. Single-cell sorting for gene expression profiling using flow cytometry has been described elsewhere (18).

SuperScript III RT (Invitrogen) was used for RT. Lysed single cells in 6.5  $\mu$ l water containing 0.5 mM dNTP (Sigma-Aldrich), 5.0  $\mu$ M oligo(dT)<sub>15</sub> (Invitrogen) and 5.0  $\mu$ M random hexamers (Invitrogen) were incubated at 65°C for 5 min; 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 20 U RNaseOut and 100 U SuperScript III (all Invitrogen; final concentrations) were added to a final volume of 10  $\mu$ l. RT was performed at 25°C for 5 min, 50°C for 60 min, 55°C for 10 min and terminated by heating to 70°C for 15 min. All samples were diluted to 30  $\mu$ l with water before qPCR.

### qPCR

LightCycler 480 (Roche Diagnostics) was used for all qPCR measurements. To each reaction (10  $\mu$ l) containing iQ SYBR Green Supermix (Bio-Rad) and 400 nM of each primer (Eurofins MWG Operon), we added 2–4  $\mu$ l of

diluted cDNA. Primer sequences used are listed in [Supplementary Table S1](#). All primers were designed with Primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and Netprimer (Premier Biosoft International). The temperature profile was 95°C for 3 min followed by 50 cycles of amplification (95°C for 20 s, 60°C for 20 s and 72°C for 20 s). The formation of expected PCR products was confirmed by agarose gel electrophoresis. All samples were analyzed by melting curve analysis. cDNA concentrations were determined by qPCR relative to standard curves based on purified PCR products (MinElute PCR Purification Kit, Qiagen). The concentration of purified PCR products was determined spectroscopically (NanoDrop ND-1000, Nanodrop Technologies). qPCR data were analyzed as described (19). Limit of detection was determined for all single-cell assays by serial dilution of known cDNA copy numbers. Six replicates were analyzed at each concentration and level of detection was determined by the lowest cDNA copy number where all six replicates were positive ([Supplementary Table S1](#)). All data points below the limit of detection were excluded from further analysis. Potential reference genes for cell population data were evaluated using NormFinder. Cell population data were normalized against the geometric mean expression of *Gapdh* and *B2m* using assay specific PCR efficiencies (20).

### Single-cell analysis

The number of genes that can be analyzed in a single cell is limited by the number of transcripts of the studied genes. Theoretically, only one molecule is needed for detection, but ~20 target molecules per PCR are needed for accurate quantification (21). This requirement was fulfilled for most of the cells and genes analyzed in this study. All single-cell assays were optimized to be specific enough not to produce primer-dimer signals within 45 cycles of amplification. Highest reproducibility is achieved by minimizing the dilution between RT and qPCR and avoiding the usage of replicates (21). Data are shown as the number of cDNA molecules per cell. The RT efficiency is gene dependent and generally <100% (22). Hence, the number of cDNA molecules is a lower-limit estimate of the number of mRNA molecules that were present in the cell. Since cDNA is single-stranded, we subtracted one cycle from the measured value when calibrating against standard curves based on double-stranded PCR products (23). Most assays were designed to span introns ([Supplementary Table S1](#)). All assays were checked by BLAST for pseudogenes. Only *GS* revealed two potential pseudogenes. All single-cell assays were tested for amplification of genomic DNA. Five individual cells per assay were tested and no genomic DNA amplification was observed.

### Statistical analysis

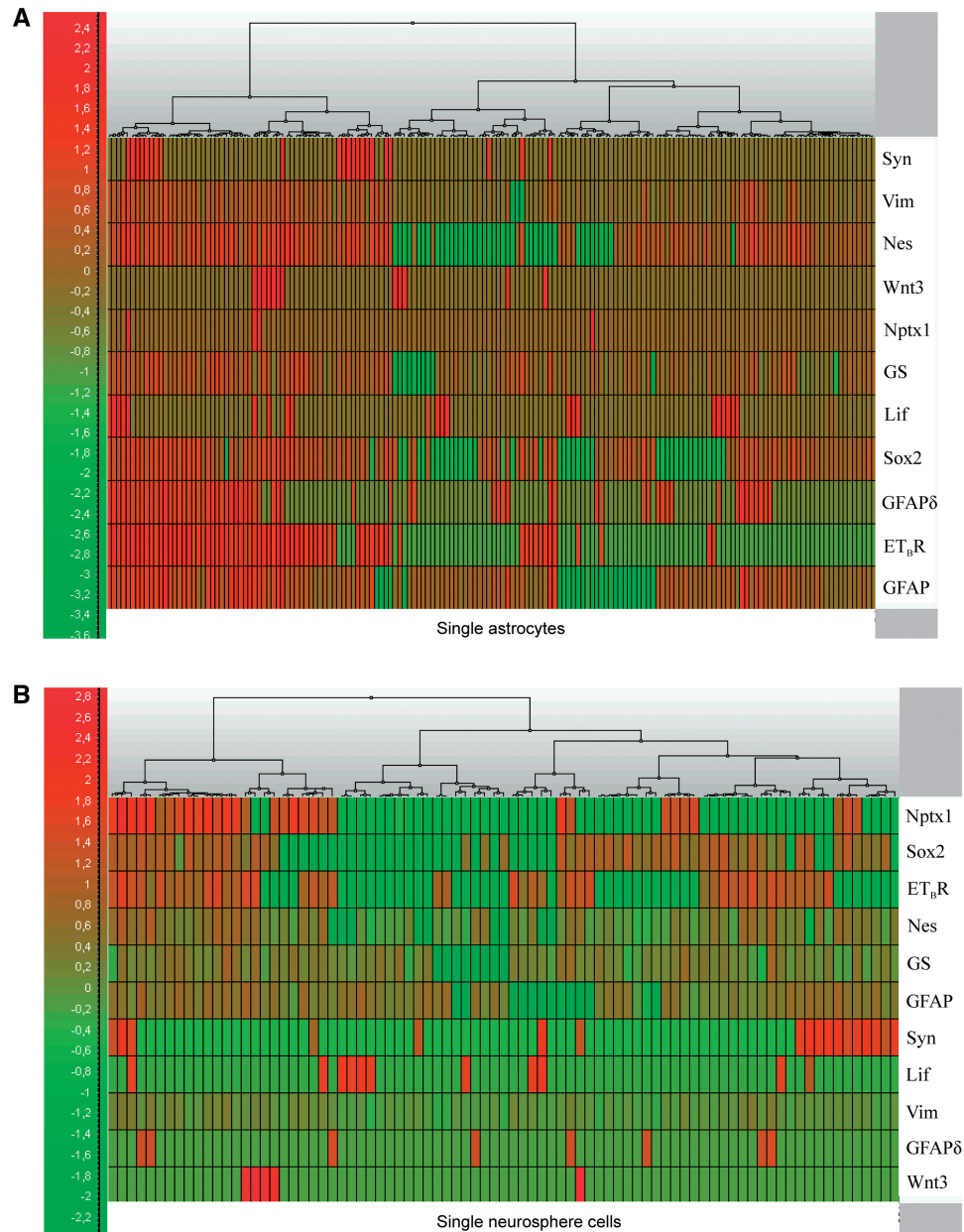
Spearman correlation and partial correlation calculations were performed in SPSS (16.0 or later, SPSS Inc.) software. We calculated first-order partial correlations for all observed Spearman correlations. Heat maps, principal component analysis (PCA), Kohonen self-organizing maps

(SOM) and potential curve analysis were performed in GenEx software (MultiD). Expression of each gene was mean-centered for the heat map analysis, which was calculated using Ward's algorithm and Euclidean distance measure. For Kohonen SOMs and potential curve analysis autoscaled gene expression values were used to give all genes equal weight in the clustering algorithms. Parameters for the Kohonen SOMs were: 2–3 × 1 map, 0.10 learning rate, 2–3 neighbors and 10 000 iterations. The resulting clusters did not depend on parameter settings. The data were analyzed as described (24). Neurosphere cells were classified by potential curve analysis using the two subpopulations of astrocytes as training set (25). The *t*-tests and Fisher's tests were performed with Bonferroni correction for multiple testing.

## RESULTS

Primary astrocyte cultures prepared from P1 mouse brains and neurospheres from P4 brains are routinely used in many experimental paradigms. Single cells were collected by flow cytometry, lysed and analyzed by RT-qPCR. Expression of *glutamine synthase (GS)*, *glial fibrillary acidic protein α (GFAP)*, *GFAPδ*, *nestin (Nes)*, *vimentin (Vim)*, *synemin (Syn)*, *SRY-box containing gene 2 (Sox2)*, *endothelin type B receptor (ET<sub>B</sub>R)*, *wingless-related MMTV integration site 3 (Wnt3)*, *leukemia inhibitory factor (Lif)* and *neuronal pentraxin 1 (Nptx1)* was profiled in 164 astrocytes and 83 neurosphere cells (Figure 1). A second set of 164 astrocytes was measured and analyzed independently; the results were comparable to those in the first set ([Supplementary Tables S2 and S3](#) and [Supplementary Figures S2 and S4](#)). To characterize individual cells we performed descriptive statistics, correlation studies and subpopulation screens using unsupervised learning algorithms.

To characterize the purity of the cultures of primary astrocytes and neurospheres, we compared the gene expression profiles with those of brain tissue from P1, P4 and P60 mice at cell population level ([Supplementary Figure S1](#)). In primary astrocyte enriched cultures, markers for microglia [*allograft inflammatory factor 1 (Aif1/Iba1)*] (26), oligodendrocytes [*myelin basic protein (Mbp)*] (27,28) and *2'-3'-cyclic nucleotide 3' phosphodiesterase (Cnp)*] (27,28), neurons [*microtubule-associated protein 2 (Mtap2)*] (29,30), *neurofilament, light polypeptide (Nefl)*] (28,31) and *Nptx1*] (32) and endothelial cells [*platelet/endothelial cell adhesion molecule 1 (Pecam1)*] (28,33), *von Willebrand factor homolog (Vwf)*] (28,33) and *angiopoietin 2 (Angpt2)*] (33) were downregulated, while markers for astrocytes [*GFAP*] (2,28,34,35), *aldehyde dehydrogenase 1 family, member L1 (Aldh1l1)*] (28) and *GS*] (36,37) were upregulated compared to mature P60 brains. These data confirm that our primary astrocytes were enriched for astrocytes and astrocyte progenitors. The expression profile of neurospheres was more complex with high expression of some but not all astrocyte (*Aldh1l1*), neuronal (*Mtap2* and *Nptx1*) and endothelial cell (*Angpt2*) markers, indicating that neurospheres are a more heterogeneous cell population.



**Figure 1.** Cell heterogeneity among primary astrocytes and neurosphere cells. Heat maps for 164 primary astrocytes (A) and 83 primary neurosphere cells (B) were constructed using Ward's algorithm and Euclidean distance measure for all cells. Expression levels of all genes were mean-centered.

#### Most cells in primary astrocyte cultures and neurosphere cells co-express astrocyte and stem cell markers

Glutamine synthase converts the neurotransmitter glutamate into glutamine and is expressed by astrocytes in the brain (36,37). Of 164 cells from primary astrocyte cultures, 153 (93%) expressed *GS* (Table 1). Among *GS*-positive cells, 78% expressed *Nes*, 75% expressed *Sox2* and 65% expressed *Nes* and *Sox2*, both markers of stem/progenitor cells (38) and found also in some astrocytes (39,40). The intermediate filament protein genes *GFAP* and *Vim* were expressed by 139 (85%) and 161 (98%) cells, respectively.

Among neurosphere cells, 90% expressed *GS*; of these, 91% expressed *Nes* and 63% expressed *Sox2*. All cells that

expressed *GS* and *Sox2* also expressed *Nes*. *GFAP*, which is expressed in astrocytes and some progenitor cells (28,34,35), was expressed in 86% of the cells—the same proportion as in astrocyte cultures. All neurosphere cells expressed *Vim*.

*GFAP $\delta$* , a splice form of *GFAP* found in neural progenitor cells (41), was expressed in 38% of primary astrocytes but in only 10% of neurosphere cells. Among *GFAP $\delta$* -positive primary astrocytes, 92% also expressed *GFAP*. *Syn*, coding for the intermediate filament protein synemin, was expressed in 13% of astrocytes and 22% of neurosphere cells. *Lif*, a factor important for stem cell self-renewal and astrocyte differentiation (42), was expressed in 13% of primary astrocytes and 13% of

**Table 1.** Statistical parameters describing gene expression in 164 primary astrocytes and 83 neurosphere cells

Gene	Cell type	$n^a$	Arithmetic mean <sup>b</sup>	Geometric mean <sup>c</sup>	Log <sub>10</sub> geometric mean (SD)	Maximum expression <sup>d</sup>
<i>GS</i>	A	153	520	320	2.5 (0.40)	2700
	NS	75	300	250	2.4 (0.26)	1100
<i>GFAP</i>	A	139	2900	1000	3.0 (0.56)	45 000
	NS	71	640	560	2.7 (0.25)	1600
<i>GFAP<math>\delta</math></i>	A	63	110	79	1.9 (0.38)	720
	NS	8	33	33	1.5 (0.07)	40
<i>Vim</i>	A	161	7500	5000	3.7 (0.38)	48 000
	NS	83	2600	2500	3.4 (0.15)	5600
<i>Nes</i>	A	124	460	320	2.5 (0.41)	3700
	NS	74	260	170	2.3 (0.42)	1200
<i>ET<sub>B</sub>R</i>	A	70	390	320	2.5 (0.35)	1600
	NS	44	240	200	2.3 (0.30)	750
<i>Sox2</i>	A	122	160	130	2.1 (0.34)	560
	NS	52	160	130	2.1 (0.25)	380
<i>Nptx1</i>	A	4	120	130	2.1 (0.10)	140
	NS	31	430	230	2.4 (0.40)	2700
<i>Wnt3</i>	A	12	200	200	2.3 (0.13)	340
	NS	5	670	630	2.8 (0.17)	1100
<i>Syn</i>	A	22	130	100	2.0 (0.39)	650
	NS	18	130	110	2.0 (0.34)	280
<i>Lif</i>	A	21	110	100	2.0 (0.31)	340
	NS	11	97	87	1.9 (0.24)	160

A, astrocytes; NS, neurosphere cells.

<sup>a</sup>Number of cells expressing a given gene.

<sup>b</sup>The arithmetic mean was calculated as:  $\mu_A = (\sum X_n)/n$ .

<sup>c</sup>The geometric mean was calculated as:  $\mu_G = (\prod X_n)^{1/n}$ .

<sup>d</sup>Highest number of cDNA molecules of a gene in any cell.

neurosphere cells, and *Wnt3*, another regulator of self-renewal and neurogenesis (43), in 7 and 6%, respectively. The most prominent difference between the cultures was in the expression of *Nptx1* that was found in 2.4% of astrocytes and 37% of neurosphere cells. We conclude that the majority of primary astrocytes and neurosphere cells co-express common astrocyte and stem/progenitor markers.

### Transcript levels in primary astrocytes and neurosphere cells show lognormal features

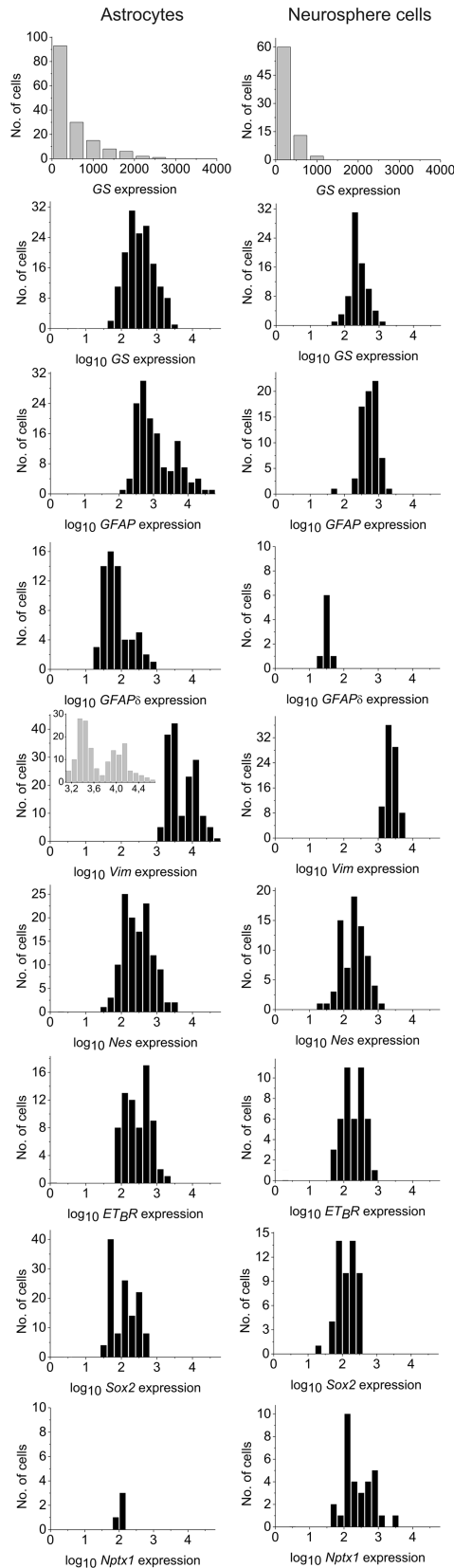
The distributions of *GS*, *GFAP*, *GFAP $\delta$* , *Vim*, *Nes*, *ET<sub>B</sub>R*, *Sox2* and *Nptx1* transcripts are shown in Figure 2 (see Supplementary Figure S2 for the second data set). Except for *Vim*, the distributions showed lognormal features, as described in other studies of mammalian cells (3,15,44). *Lif*, *Syn* and *Wnt3* transcripts were detected in only few cells (Table 1 and Supplementary Table S2) and thus their distributions could not be reliably determined. The geometric and arithmetic means of the expression of individual genes are shown in Table 1. In a lognormal population, the geometric mean reflects the characteristic expression in a typical/median cell. The geometric mean is more conservative than the arithmetic mean. The latter overestimates the characteristic expression when expression levels are lognormally distributed.

The variations in gene expression levels between individual cells were substantial. Some astrocytes had ~50 000 transcripts of *Vim* and *GFAP* per cell, while others had fewer than 100. The observed transcript variability is in

agreement with transcriptional bursting (15,16). A consequence of the observed lognormality is that a majority of the transcripts for a particular gene originate from a minority of the cells in a given population. For example, the 30 primary astrocytes with highest number of *GFAP* transcripts contributed to ~75% of all transcripts for this gene.

### Expression of genes upregulated in activated astrocytes correlates at the cellular level

Next, we looked for correlations between the mRNA levels of multiple genes in each cell. Table 2 shows Spearman correlation coefficients for all gene pairs. The correlation coefficient is a value between -1 and 1, where 1 reflects perfect positive correlation, -1 reflects negative correlation and 0 indicates no correlation. Interestingly, genes that are upregulated in activated astrocytes (*GFAP*, *Vim*, *Nes*, *GS* and *ET<sub>B</sub>R*) (45–49) showed positive correlations ( $P < 0.01$ ) in individual cells collected from primary astrocyte cultures (Table 2). Also, expression of *Sox2* and to some degree *GFAP $\delta$*  correlated with the expression of the intermediate filament genes *GFAP*, *Vim* and *Nes*, as well as with the expression of *GS* and *ET<sub>B</sub>R* ( $P < 0.01$ ). The only negatively correlation observed was between *Sox2* and *Wnt3*. In neurosphere cells, however, these genes showed little or no correlation, except for *Vim*, whose expression correlated with *Nes* and *ET<sub>B</sub>R*. In contrast, neurosphere cells expressed high levels of *Nptx1*, whose expression correlated positively with that of *Sox2*.



**Figure 2.** Gene expression levels in 164 primary astrocytes and 83 neurosphere cells. Gene expression is shown as the number of cDNAs per cell. *GS* expression is shown in both linear and  $\log_{10}$  scales; other genes are shown in  $\log_{10}$  scale. Inset shows a more detailed histogram of *Vim* expression in astrocytes.

To discriminate between direct and indirect interactions among the observed correlations, we calculated partial correlations between gene expression levels in individual cells. This was done by specifying a control gene that may interact with two other correlated genes and thus account for the observed correlation (50). The resulting partial correlation then becomes a unique correlation between the two initial genes that remains when the correlated variance explained by the control gene has been removed. Using partial correlations, we could determine if a measured correlation between two genes was unique or rather a consequence of the two genes both being dependent on a third gene (Figure 3A). Figure 3B shows the gene interaction map for *Vim* based on partial correlations. *Vim* interacts directly with *ET<sub>B</sub>R* and *Nes*. These interactions are independent of the other genes studied. The *Vim* interactions with *GS*, *GFAP* and *Sox2* were partially direct, while its interaction with *GFAP $\delta$*  was indirect and could be explained as being a consequence of *Vim*'s interaction with either *GFAP* or *ET<sub>B</sub>R*. Interaction maps for the other genes are shown in Supplementary Figure S3. From the 20 statistically significant correlations in Table 2, nine interactions were direct while eleven could be fully explained by other genes using partial correlations. All direct correlations were dependent on *Vim* except for those that involved *GFAP $\delta$*  and the interaction between *Sox2* and *Wnt3*. Figure 3C shows the complete interaction map based on the correlations in Table 2.

### Primary astrocytes can be divided into two subpopulations

To identify possible subpopulations of cells based on their expression profile, we applied Kohonen SOMs (Figure 4A). SOM is an unsupervised learning algorithm that divides the cells into a given number of groups based on their characteristics. SOM uses random numbers to initiate and perform the classification. As a consequence reiterated SOM analysis may generate different classifications. If the same SOM is repeatedly produced, it evidences robust classification. The classification depends on gene expression levels. Highly expressed genes have greater influence than lowly expressed genes. This effect can be removed by subtracting the average of the expression level of each gene and dividing it by its standard deviation, i.e. performing autoscaling (24).

The astrocytes were divided into two groups using a  $2 \times 1$  SOM (Figure 4A). The SOM was based on the autoscaled expression levels of all eleven genes and was fully reproducible. The SOM classification was confirmed using principal component analysis (PCA), another unsupervised classification method based on different principles (Figure 4B) (24). To characterize the two subpopulations, we plotted the transcript distributions of the highly expressed genes: *Vim*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes*, *Sox2* and *ET<sub>B</sub>R* (Figure 4C). This analysis revealed two, albeit overlapping, lognormal distributions. In the first subpopulation *Vim*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes*, *Sox2* and *ET<sub>B</sub>R* were upregulated two- to five-fold ( $P < 0.01$ , Table 3) relative to the second subpopulation. In addition, more cells expressed *GFAP $\delta$* , *Syn* and *ET<sub>B</sub>R* in the first subpopulation ( $P < 0.01$ ). No significant

**Table 2.** Spearman correlation coefficients for primary astrocytes and neurosphere cells

Gene	Cell type	<i>GS</i>	<i>GFAP</i>	<i>GFAP<math>\delta</math></i>	<i>Vim</i>	<i>Nes</i>	<i>ET<sub>B</sub>R</i>	<i>Sox2</i>	<i>Nptx1</i>	<i>Wnt3</i>	<i>Syn</i>	<i>Lif</i>
<i>GS</i>	A	1										
	NS											
<i>GFAP</i>	A	<b>0.51</b>	1									
	NS	0.05										
<i>GFAP<math>\delta</math></i>	A	<b>0.35</b>	<b>0.57</b>	1								
	NS	0.09	0.26									
<i>Vim</i>	A	<b>0.59</b>	<b>0.56</b>	<u>0.23</u>	1							
	NS	0.21	0.08	<u>0.15</u>								
<i>Nes</i>	A	<b>0.50</b>	<b>0.39</b>	0.06	<b>0.68</b>	1						
	NS	0.08	0.19	0.47	<b>0.66</b>							
<i>ET<sub>B</sub>R</i>	A	<b>0.24</b>	<b>0.29</b>	<u>0.25</u>	<b>0.57</b>	<b>0.29</b>	1					
	NS	0.13	0.04	<u>0.15</u>	<b>0.47</b>	0.28						
<i>Sox2</i>	A	<b>0.45</b>	<b>0.44</b>	0.19	<b>0.56</b>	<b>0.38</b>	<b>0.37</b>	1				
	NS	-0.03	0.28	0.06	0.20	0.10	-0.05					
<i>Nptx1</i>	A	-0.33	-0.43	-	0.43	0.60	0.310	0.60	1			
	NS	0.06	-0.01	-	0.35	0.15	0.05	<u>0.45</u>				
<i>Wnt3</i>	A	-0.11	-0.40	0.19	-0.28	-0.34	0.18	<b>-0.67</b>	-	1		
	NS	-0.50	-	-	0.05	0.30	-	-0.20	-			
<i>Syn</i>	A	0.01	0.12	-0.22	-0.03	0.10	0.07	0.12	-	-	1	
	NS	-0.03	0.42	-	-0.02	0.09	-0.01	-0.10	-0.11	-		
<i>Lif</i>	A	0.23	-0.10	-0.16	0.23	0.22	0.13	0.37	-	-	-	1
	NS	0.03	0.38	-	-0.39	0.17	-0.71	-	-	-	-	

All cells (328 single astrocytes and 83 dissociated neurosphere cells) were used for correlation calculations. Bold indicates  $\geq 99\%$  significance; underscore indicates  $\geq 95\%$  significance. Correlation coefficients were not calculated for gene pairs with fewer than five data points. A, astrocytes; NS, neurosphere cells.

differences for *Lif*, *Nptx1* and *Wnt3* were observed. The presence of two astrocyte subpopulations was confirmed in the independent data set (Supplementary Figure S4). The SOM analysis of the neurosphere cells revealed no distinguishable subpopulations (data not shown).

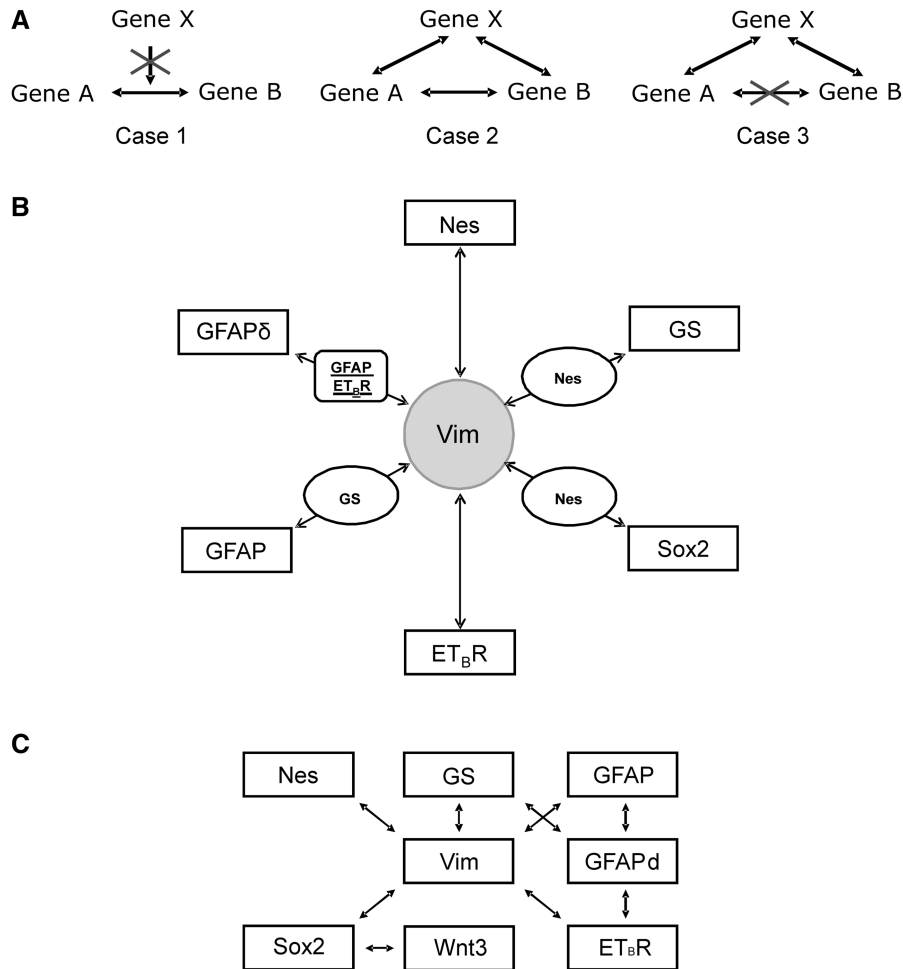
It was not possible to identify subpopulations of primary astrocytes based on the presence or absence of expression of any unique marker. All genes showed a unimodal distribution of transcript levels, except for *Vim* transcript levels, which had a bimodal distribution (Figure 2), implying two subpopulations of cells. Next, we tested if *Vim* can be used as a single classifier for the two subpopulations by indexing the cells using a threshold of 6300 transcripts, placed centrally between the two peaks in the distribution (Figure 2). Out of 164, 155 of astrocytes were accurately classified using *Vim* as a classifier (Supplementary Figure S5). Clearly, the *Vim* expression level is highly characteristic for these two astrocyte subpopulations. To test if *Vim* is the sole determinant we excluded it from the analysis. The resulting SOM classification correctly classified 159 of 164 astrocytes (Supplementary Figure S6). Furthermore, we also tested if the expression data support the presence of three astrocyte subpopulations using a  $3 \times 1$  SOM (Supplementary Figure S7). Again, two of the groups were characterized by an overall high and low expression, respectively of *Vim*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes*, *Sox2* and *ET<sub>B</sub>R*. The new third group had an intermediate expression pattern relative the other two, but most similar to the low expressing group. The intermediate group showed no unique features, suggesting the existence of only two distinct subpopulations of astrocytes. This conclusion was supported by PCA (Supplementary Figure S7B).

Finally, we determined if the neurosphere cells had gene expression profile similar to any of the astrocyte subpopulations using PCA and potential curve analysis (Figure 5). The principal component space was calculated using the expression profiles of the astrocytes only. When the neurosphere cells were positioned in this space based on their expression profiles, the classification revealed a high degree of similarity between gene expression profile of neurosphere cells and the astrocyte subpopulation characterized by low expression of *Vim*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes*, *Sox2* and *ET<sub>B</sub>R*.

## DISCUSSION

Most single-cell gene expression studies in the brain have focused on neurons and neuronal progenitors (4–7,11, 51,52). Here, we characterized primary astrocytes and neurosphere cells. Primary astrocyte cultures prepared from neonatal rodent brains have long served as an experimental system to study the properties of astrocytes (53). These cultures are derived from unidentified populations of proliferating precursor cells. Comparative microarray experiments have shown that primary astrocytes are similar, but not identical, to *in vivo* astrocytes (28). Here, we studied eleven different markers selected to reflect astrocyte properties, including astrocyte activation and stem/progenitor cell properties.

Our analysis of primary astrocytes showed prominent expression of established markers for astrocytes, while the expression of other cellular markers was low (Supplementary Figure S1). At a single-cell level, 94% of astrocytes expressed *GS* (Table 1), a marker of immature and mature astrocytes (36). Low expression of *GS* has also



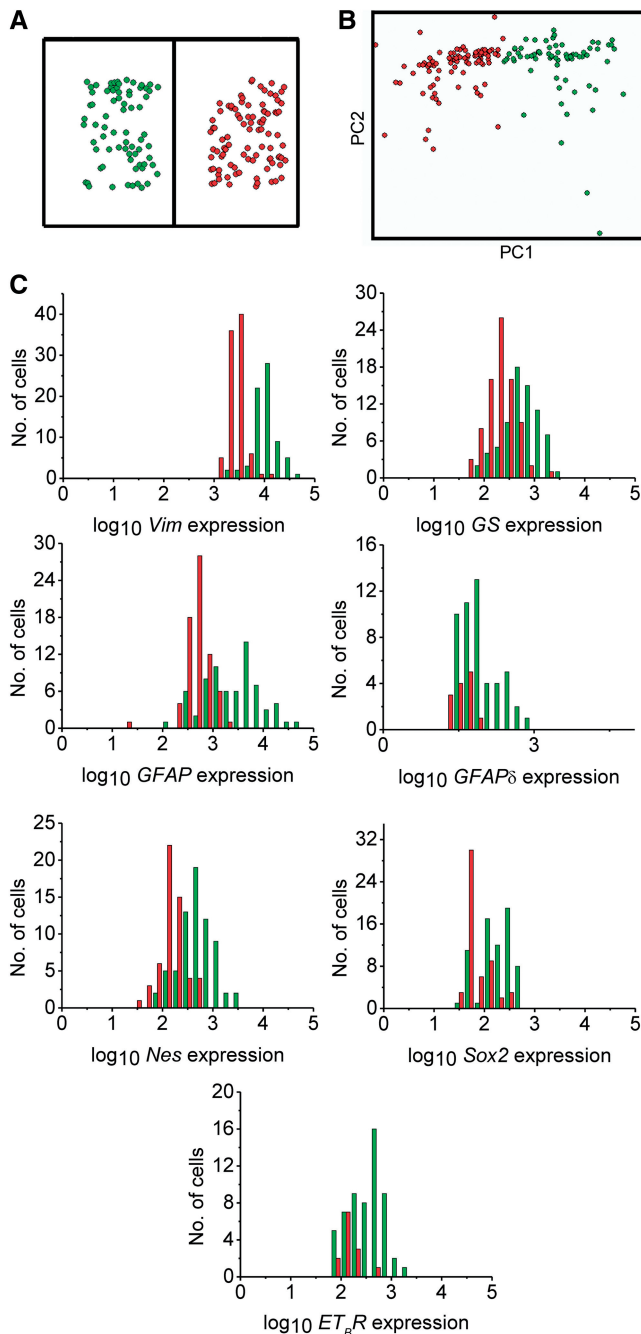
**Figure 3.** Gene interactions. (A) Three different types of interaction between two genes can be identified using partial correlations. Case 1 shows a direct interaction between genes A and B. Case 2 represents a direct interaction that can be partly explained by a third gene, while case 3 represents an indirect interaction that can be fully explained by a third gene. We used a decrease of 0.15 in correlation as a cut off for partially explained interactions (Case 2) and a complete loss of significance for indirect correlation (Case 3). (B) A detailed interaction map for *Vim*. The interaction between *Vim* and *Nes/ET<sub>B</sub>R* is direct (Case 1), while the interactions with *GFAP*, *GS* and *Sox2* can be partially explained by other genes (*GS* and *Nes*, Case 2). The interaction between *Vim* and *GFAP* is indirect and can be fully explained by interactions through *GFAP* or *ET<sub>B</sub>R*. See [Supplementary Figure S3](#) for detailed interaction maps for other genes. (C) Nine of 20 observed correlations in Table 2 represented direct interactions that could not be explained by the other genes.

been reported in oligodendrocytes (37). *GFAP* has lower cell type specificity than *GS* but is not expressed in mature oligodendrocytes (28,34). Seventy nine percent of cells in astrocyte cultures co-expressed *GFAP* and *GS*. Furthermore, 69 of 70 *ET<sub>B</sub>R*-positive cells in astrocyte cultures co-expressed *GS*. Thus, since endothelial cells are *ET<sub>B</sub>R*-positive and *GS*-negative (54), our primary astrocyte cultures were not contaminated with endothelial cells. Only four cells in astrocyte cultures expressed *Nptx1*, a marker for neuron and neural progenitor cells (32). Our study shows that most cells in primary astrocyte cultures co-express the astrocyte marker *GS* and two genes expressed by neural progenitor/stem cells, namely *Nes* and *Sox2*. In summary, the primary astrocytes have expression pattern similar to that of stem/progenitor cells and astrocytes *in vivo*. However, we cannot exclude the possibility that other cell types exist in our cultures, but they should be present only in small fractions.

Cell population data for the neurospheres showed that these cells have less distinct expression pattern than the primary astrocytes ([Supplementary Figure S1](#)). Markers for endothelial cells (*Angpt2*) and neurons (*Nptx1* and *Mtap2*) were highly expressed in neurospheres, while markers for astrocytes (*Aldh1l1*, *GFAP* and *GS*) were expressed to a lesser degree compared to primary astrocytes. Interestingly, 78% of the neurosphere cells co-expressed *GFAP* and *GS* at a single-cell level. Thirty seven percent of the neurosphere cells expressed *Nptx1*. Similar to the primary astrocytes, the majority of the neurosphere cells co-expressed *GS*, *Sox2* and *Nes*. In summary, the neurosphere cells have some properties similar to primary astrocytes. They also show characteristics of several cell types, a finding consistent with the fact that they are a heterogeneous cell population.

High correlation between the numbers of transcripts per cell of *GFAP*, *Vim*, *Nes*, *GS* and *ET<sub>B</sub>R* suggests that





**Figure 4.** Astrocyte subpopulations show distinct gene expression profiles. (A) Clustering of astrocyte subpopulations using Kohonen SOMs. Expression levels of all genes were autoscaled. Each dot represents one cell. (B) Principal component analysis confirmed the existence of two subpopulations with coloring according to the Kohonen SOMs classification. (C) Histograms of gene expression profiles (log<sub>10</sub> scale) of the two astrocyte subpopulations. Descriptive statistics for the two astrocyte populations are shown in Table 3. PC, principal component.

these genes have common regulatory elements and might be transcribed in synchronized bursts (55). Indeed, they are all known to be upregulated in activated astrocytes (45–49). Surprisingly, the expression of a stem cell marker, *Sox2* (38), correlated positively with the

expression of intermediate filament proteins *GFAP*, *Vim* and *Nes*, as well as with *GS* and *ET<sub>B</sub>R* in the primary astrocytes. Thus, the activation of astrocytes may be linked to a transition into a more immature or stem cell-like state, as suggested by studies reporting that at least some astrocytes acquire stem cell properties after brain injury (56). Interestingly, all observed correlations were directly dependent on either *Vim* or *GFAP $\delta$* , except the negative correlation between *Sox2* and *Wnt3*. These data suggest that *Vim* and *GFAP $\delta$*  may have important role in cell fate determination in primary astrocytes (Figure 3C).

The bimodal distribution of *Vim* expression was apparent already from inspection of raw data, but this was not the case for the other genes (Figure 2). Only after cells were classified into two subpopulations did bimodal expression profiles become evident for *ET<sub>B</sub>R*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes* and *Sox2* (Figure 4C). The distribution of gene expression levels among individual cells in the respective subpopulations was almost perfectly lognormal. Conventional cell type characterizations are generally based on presence or absence of well-established markers. This was not possible here, since no such marker is known. Instead, we applied multivariate methods to divide samples into groups. Using SOM and PCA analyses we were able to show that the primary astrocyte cultures are a mixture of two defined subpopulations with unique expression profiles. The most distinct single classifier, *Vim*, is expressed in both subpopulations, although to partially different levels. Based on *Vim* transcript levels alone, 95% of the primary astrocytes could be correctly classified. A drawback of using *Vim* alone as a classifier is that the threshold value is variable between different cell populations: 6300 and 2500 *Vim* transcripts, respectively were used in the two independent data sets. We do not know the underlying reason for the different thresholds. We conclude that for subpopulation classification, the use of multivariate SOM analysis is more accurate and robust than the use of *Vim* expression alone.

The two subpopulations of cells in primary astrocyte cultures may represent different cell states or different cell types, which may be reversible. From our data, we cannot discriminate between these alternatives. However, classification of astrocytes into three groups showed cells with an intermediate expression profile. It is conceivable that these astrocytes are in a transition state between the original two subpopulations. This would support the hypothesis of reversible cell states. Such subpopulations may also exist in the brain or be the result of *in vitro* culture conditions. *In vivo* studies have revealed different subpopulations of astrocytes (57,58)—none similar to those described here—classified by the expression or lack of expression of specific markers identified by immunostaining or in some cases by electrophysiological properties (58,59). We identified subpopulations of primary astrocytes not by the presence of specific markers but rather by expression levels of shared markers. Conceivably, the subpopulation of astrocytes with high transcript levels correspond to activated astrocytes *in vivo*, which are characterized by upregulation of

*GFAP*, *Nes*, *ET<sub>B</sub>R* and *Syn* (48,60). The gene expression profile of astrocytes changes during brain development (Supplementary Figure S1) (28,61). We found that *ET<sub>B</sub>R*, *GS*, *GFAP*, *GFAP $\delta$* , *Nes*, *Sox2*, *Vim* were co-regulated at

**Table 3.** Statistical profile of subpopulations in primary astrocytes

Gene	Statistics <sup>a</sup>	Low expressing cells <sup>b</sup>	High expressing cells <sup>b</sup>	Ratio <sup>c</sup>
<i>Vim</i>	<i>N</i>	89	72	<b>4.3</b>
	Geometric mean	2600	11 000	
<i>GS</i>	<i>N</i>	81	72	<b>2.9</b>
	Geometric mean	210	600	
<i>GFAP</i>	<i>N</i>	70	69	<b>5.0</b>
	Geometric mean	500	2500	
<i>GFAP<math>\delta</math></i>	<i>n</i>	13	<b>50</b>	<b>2.4</b>
	Geometric mean	35	86	
<i>Nes</i>	<i>n</i>	55	69	<b>3.5</b>
	Geometric mean	140	510	
<i>ET<sub>B</sub>R</i>	<i>n</i>	13	<b>57</b>	<b>2.3</b>
	Geometric mean	150	330	
<i>Sox2</i>	<i>n</i>	53	69	<b>2.4</b>
	Geometric mean	73	180	
<i>Wnt3</i>	<i>n</i>	4	8	0.81
	Geometric mean	220	180	
<i>Syn</i>	<i>n</i>	3	<b>19</b>	1.3
	Geometric mean	74	96	
<i>Lif</i>	<i>n</i>	12	9	1.1
	Geometric mean	89	100	

<sup>a</sup>*n*, Number of cells expressing a given gene in the subpopulation defined by the two groups. *Nptx1* was excluded because it was expressed by only four cells. Bold numbers indicate that the total number of cells among the cells with high expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* and *Sox2* was increased compared to the cells with low expression. ( $P < 0.01$ , Fisher's exact test with Bonferroni correction).

<sup>b</sup>Subpopulations defined by low/high expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* and *Sox2*.

<sup>c</sup>Ratio of expression between cells with high and low expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* for a given gene in astrocytes. Bold numbers are statistically significant ( $P < 0.01$ , *t*-test with Bonferroni correction).

single-cell level *in vitro*, whereas *GS*, *GFAP* and *GFAP $\delta$*  were upregulated and *ET<sub>B</sub>R*, *Nes*, *Sox2* and *Vim* were downregulated in developing brains (Supplementary Figure S1) (61). Thus, the two subpopulations of cultured astrocytes are unlikely to reflect different stages of maturation.

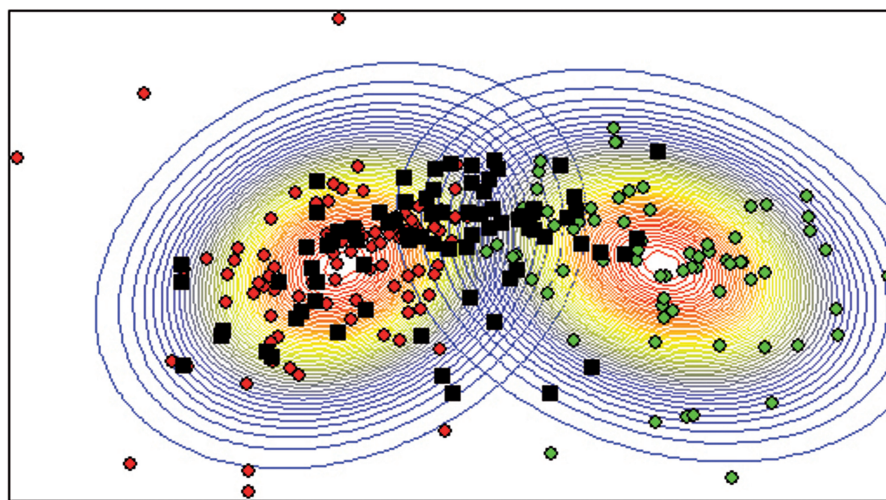
In summary, we introduce how single-cell gene expression profiling can be applied as a novel research tool to identify and characterize distinct subpopulations of cells and how gene correlations can be applied to determine detailed gene interaction networks using this tool. We found that the majority of cells in primary astrocyte cultures and cells from dissociated neurospheres express mRNAs encoding markers characteristic of astrocytes as well as markers characteristic of neural stem/progenitor cells. The transcription of genes encoding proteins associated with astrocyte activation seems to be regulated by a common mechanism where *Vim* and *GFAP $\delta$*  have key functions. The population with high expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* and *Sox2*, has the gene expression profile of activated astrocytes, while the population with low expression has a profile similar to neurosphere cells.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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**Figure 5.** Classification of neurosphere cells. Principal component and potential curve analysis were used to classify neurosphere cells (black) based on the expression profiles of the two subpopulations of astrocytes. Each dot represents one cell: red dots are cells with low expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* and *Sox2*; green dots are cells with high expression of *Vim*, *GFAP*, *GFAP $\delta$* , *Nes*, *ET<sub>B</sub>R* and *Sox2*.

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