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# Don't Look: Growing Clonal Versus Nonclonal Neural Stem Cell Colonies

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## ABSTRACT

**Recent reports have challenged the clonality of the neurosphere assay in assessing neural stem cell (NSC) numbers quantitatively. We tested the clonality of the neurosphere assay by culturing mixtures of differently labeled neural cells, watching single neural cells proliferate using video microscopy, and encapsulating single NSCs and their progeny. The neurosphere assay gave rise to clonal colonies when using primary cells plated at 10 cells/$\mu$l or less; however, when using passaged NSCs, the spheres were clonal only if plated at 1 cell/$\mu$l. Most important, moving the plates during the growth phase (to look at cultures microscopically) greatly increased the incidence of nonclonal colonies. To ensure clonal sphere formation and investigate nonautonomous effects on clonal sphere formation frequencies, single NSCs were encapsulated in agarose and proliferated as clonal free-floating spheres. We demonstrate that clonal neurospheres can be grown by avoiding movement-induced aggregation, by single-cell tracking, and by encapsulation of single cells.** STEM CELLS *2008;26:2938–2944*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

There are two cardinal features of stem cells: (a) they must demonstrate long-term self-renewal and (b) must be multipotential (i.e., the progeny of single stem cells must comprise multiple cell types of the tissue in which they reside). To assess the presence of stem cells many researchers have taken advantage of the in vitro proliferation of neural stem cells to form clonal colonies in either monolayer cultures [1] or a free-floating three-dimensional colony known as neurospheres [2]. Recently, the validity of the in vitro neurosphere-formation assay as a measure of clonality and multipotentiality [3–5], and as an accurate predictor of the in vivo number of stem cells in a tissue [6], has been called into question. Many laboratories have used sphere-forming assays to identify stem cells in various tissue types, including brain [2, 7–10], retina [11–13], cornea [14] olfactory neuroepithelium [15], pancreas [16], skin [17], muscle [18], bone marrow [19], and embryonic stem cells [20].

The studies [3–5] that call into question the clonality of the sphere assay also challenge the conclusions of studies in which the sphere assay was used. In most cases the number of in vitro spheres (presumed to arise from single bona fide stem cells) was assumed to predict the in vivo stem cell number and differentiation capability. We re-examined the neurosphere assay using two fluorescent-reporter mouse strains to test the clonality of the primary neurospheres derived from primary cultures of the adult forebrain subependyma, as well as from passaged neurospheres. The latter cell population was used by all three of the recent studies [3–5] questioning the clonality of the neurosphere assay. Many experimenters move their plates to the microscope repeatedly to check for sphere colony formation or refeed their cells during the neurosphere growth phase. We tested whether this movement-induced fluid motion increased the probability of aggregation and thus chimeric (nonclonal) neurosphere formation. That is, was there a significant effect of the observer changing the phenomenon studied (single cells proliferating to form a clonal neurosphere), a Heisenberg-like effect [21].

Stem cells are not isolated single cells in vivo but rather are surrounded in niches by other, more differentiated cell types. To test for paracrine effects (factors released from the surrounding cells) in vitro, an experimental design is needed that would allow the neural cells to proliferate and stay clonal in a high-cell-density in vitro environment. The technology of encapsulating stem cells in an agarose capsule was developed to study cell-cell interactions within embryonic stem cell-derived embryoid bodies [22, 23]. We encapsulated single neural stem cells in agarose capsules and tested both whether they could still proliferate into clonal neurospheres and whether there would be an effect on the encapsulated cells by a high cell density of sur-

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rounding cells. We also observed the growth of neurospheres from single neural stem cells (at very low cell densities) using time-lapse video microscopy to confirm that these cells could proliferate to form clonal neurospheres.

The present report demonstrates conditions under which neural stem cells will grow clonally in neurosphere culture, The simple movement of the cultures to observe them or add more nutrients is sufficient to destroy clonality. Finally and surprisingly, the growth of single encapsulated neural stem cells was inhibited by high cell density.

## MATERIALS AND METHODS

### Animals

The mice used were as follows: (a) double-stranded red fluorescent protein mice (adult dsRed), Tg (ACTB-dsRed*MST) 1Nagy, which is on a mixed background of (129S6/SvEvTac × C57BL/6)F1; (b) enhanced yellow fluorescent protein mice (EYFP), Tg (ACTB-EYFP) 7AC5Nagy (Jackson Laboratories, Bar Harbor, ME, http://www.jaxmice.jax.org), which is on a F129S1/SvImJ background; (c) monomeric red fluorescent protein mouse (mRFP) (actin-mRFP)1F1Hadj/J, which is on a F129S1/SvJ background; and (d) CD1 (Charles River Laboratories, Pointe-Claire, Quebec, Canada, http://www.criver.com). All animal experiments were approved by the University of Toronto Animal Care Committee, in accordance with federal laws.

### Dissection

The subependyma of the forebrain lateral ventricle was dissected out and subjected to an enzymatic digestion (1.33 mg/ml trypsin, 0.67 mg/ml hyaluronidase, and 0.2 mg/ml kynurenic acid) for 45 minutes at 37°C on a tube rocker. The cells were spun down, and the supernatant was replaced with 1 mg/ml trypsin inhibitor and resuspended in serum-free media. Cell counts and viability were assayed using trypan blue exclusion [7].

### Plating

Cells were plated at various densities (0.5–50 cells/$\mu$l, which corresponds to 131–13,158 cells per cm$^2$) in growth factor media (20 ng/ml epidermal growth factor + 10 ng/ml fibroblast growth factor-2 + 2 $\mu$g/ml heparin). YFP and dsRed cells were plated at equal densities in two sets of 24-well Nunclon plates (VWR Canlab, Burlington, Ontario, Canada, http://www.vwrcanlab.com) for the neurosphere assay. One set of plates were placed in the back of the incubator and not moved during the 7-day growth period, and the other set of plates was taken out daily and checked under the microscope. All experiments were done at least three times, and at least six wells per condition and per experiment were counted.

### Encapsulation

To encapsulate single cells within an agarose matrix, 2.0% (wt/vol) SeaPrep agarose (Cambrex, Rockland ME, http://www.cambrex.com) was prepared with phosphate-buffered saline. Methods similar to aggregate encapsulation [23] were used. Briefly, 2–4.5 × 10$^6$ NSCs were suspended in 300 $\mu$l of serum-free media, mixed with 1.2 ml of agarose and 75 $\mu$l of Pluronic F-68 (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com), and allowed to equilibrate to 37°C. Small drops of the cell suspension/agarose mixture were added to 20 ml of emulsion oil heated to 37°C (200 ml dimethylpolysiloxane; Sigma-Aldrich) in a scintillation vial using a 1-ml pipetteMan (Gilson Inc, Middleton, WI, http://www.gilson.com). The mixture was emulsified using the CellSys Microdrop Maker (One Cell Systems Inc, Cambridge, MA, http://www.onecell.com) set at 2,100 rpm for 1 minute at room temperature followed by 1 minute in an ice bath and then set at 1,100 rpm for 10 minutes in the ice bath. The encapsulation mixture was divided into two 15-ml conical tubes, and 5 ml of Hanks' balanced saline solution (HBSS; Gibco, Grand Island, NY, http://www.invitrogen.com) was overlaid prior to centrifugation at 600*g* for 10 minutes. The oil phase was removed from the top of each tube, and the aqueous phase was aspirated. Both pellets were resuspended in 2 ml of HBSS and added to 10 ml of fresh HBSS in another 15-ml conical tube prior to centrifugation at 400*g* for 5 minutes. The now encapsulated cells were resuspended in serum-free media, and then cell counts and viability were assayed using trypan blue exclusion. The cells were only used if the final single-cell encapsulated cells were more than 98% of the cells encapsulated. Finally, the single encapsulated neural stem cells and progeny were plated alone at a low density (5 cells/$\mu$l) or plated at this density with a high density (100 cells/$\mu$l) of unencapsulated dissociated host neurosphere cells (neural stem cells and their progeny). The encapsulation experiments were done three times, and four to six wells were counted per condition and per experiment.

### Passaging

Spheres were mechanically dissociated using a small-borehole fire-polished pipette, and cells were filtered through a cell strainer (BD BioScience, Mississauga, Ontario, Canada, http://www.bdbiosciences.ca) (40-$\mu$m mesh) and resuspended in serum-free media. Cell counts and viability were assayed using trypan blue exclusion. All passaging experiments were done three times, and at least six wells per condition and per experiment were counted.

### Imaging

Cells were plated at 10 cell/$\mu$l in both 6-well (3,125 cells per cm$^2$) and 24-well (2,632 cells per cm$^2$) plates and imaged every 10 minutes over the course of 7 days using a Zeiss Axiovert 200 Imaging System (Carl Zeiss, Jena, Germany, http://www.zeiss.com) [32, 33].

### Statistical Analysis

All data were compiled using Microsoft Excel (Microsoft, Redmond, WA, http://www.microsoft.com), and then statistical analysis was carried out using analysis of variance (GB STAT, Dynamic Microsystems Inc., Silver Spring, MD).

## RESULTS

### Primary Neurosphere Formation

We isolated neural stem cells from the subependymal region of adult transgenic mice expressing either YFP or dsRed protein behind the actin promoter. The single dissociated cells from the subependyma of each mouse strain were plated together at a 1:1 ratio at final cell densities ranging from 0.5 to 50 cells/$\mu$l (Fig. 1A). The cells proliferated and formed neurospheres, with a diameter of at least 100 $\mu$m, over the course of 7 days (Fig. 2A–2F). We observed that spheres formed from primary cells were clonal (derived from either dsRed alone or YFP alone) 100% of the time at 0.5 cells/$\mu$l whether the cells were left undisturbed during the growth phase of sphere formation or taken out of the incubator and observed under the microscope daily (Fig. 3A). The cells that were left undisturbed (stationary) during the experiment gave rise to clonal spheres when plated at 10 cells/$\mu$l or less, giving rise to 50–60 spheres per well, with an incidence of chimerism of only 4% at 10 cells/$\mu$l. However, if the initial cell density was increased to 20 cells/$\mu$l (more than 75 spheres per well) or if the plates were moved when the density was only 5 cells/$\mu$l or higher, the incidence of nonclonal sphere formation was always greater than 10% (Fig. 3A); similar increases in nonclonal sphere formation were found when the plates were moved only on day 3 in vitro (data not shown). There was a significant increase in the incidence of chimeric sphere formation between the stationary plates and the moved plates ($F_{1, 20} = 5.33$; $p < .05$) and with increasing cell density ($F_{4, 29} = 4.79$; $p < .05$). There was no significant interaction effect. In the experiments where the plates were moved to the

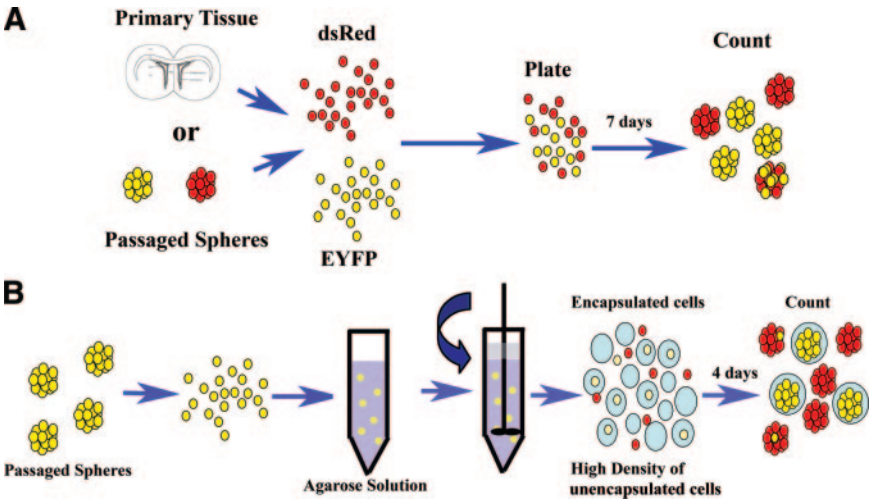


**Figure 1.** Experimental design. **(A):** Methodology for testing the clonality of both the primary and passaged neural stem cells. Briefly, the cells from the brain or passaged neurosphere cells are dissociated, plated at a 1:1 ratio, and counted 7 days later. **(B):** Basic method for the encapsulation experiment. The passaged neurospheres are dissociated, placed in an agarose solution, spun in a Microdrop Maker to encapsulate the cells, plated, and counted 4 days later.

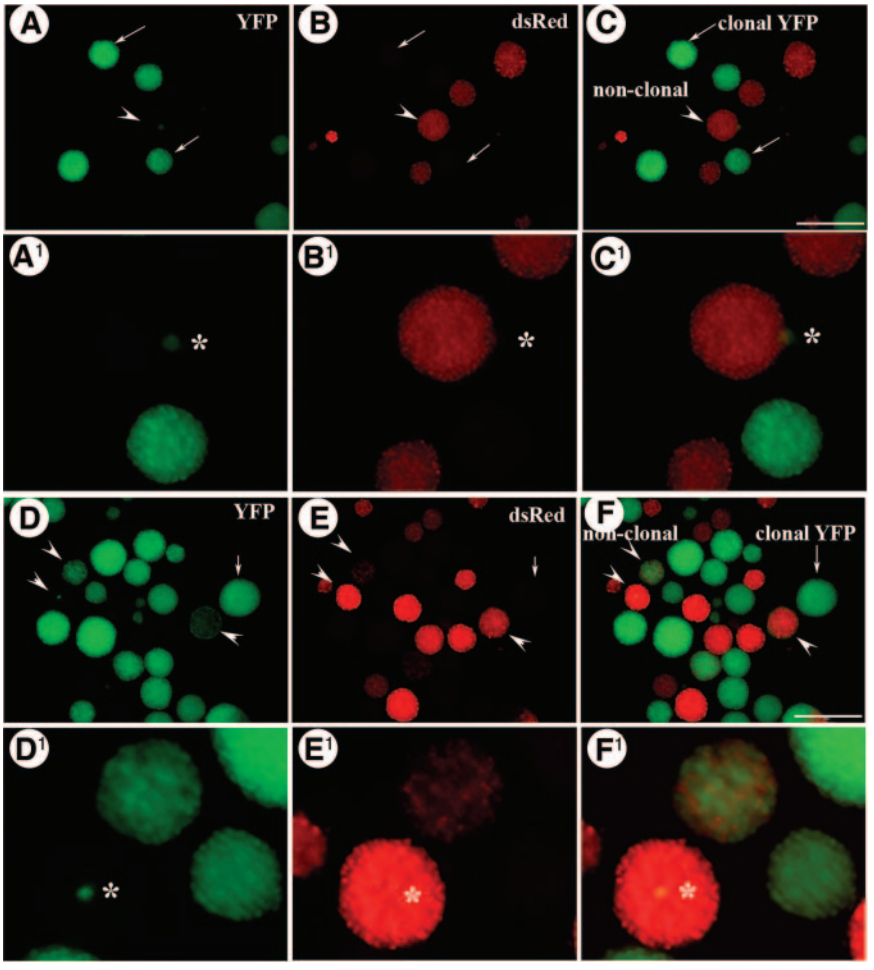


**Figure 2.** Neurosphere formation. Primary stem cells plated at 10 cells/$\mu$l in plates that were either stationary (not moved at all during the growth phase) (YFP **[A]**, dsRed **[B]**, and merged **[C]**) or moved (taken out daily and examined under the microscope) (YFP **[D]**, dsRed **[E]**, and merged **[F]**). **(A–C)** show the same field from a stationary experiment and demonstrate that the spheres were well dispersed and not touching after 7 days of growth. **($A^1$–$C^1$)** are enlargements of the one chimeric dsRed sphere with a single YFP cell attached to the outside from panels **(A–C)** (marked by white asterisks). **(D–F)** show (in a single field) that moving the plates forced the spheres closer together and increased the rate of chimerism. **($D^1$–$F^1$)** are enlargements of two chimeric spheres from **(D–F)**, one dsRed sphere with a single YFP cell on the outside of the middle of the sphere (marked by white asterisks) and one intermixed dsRed/YFP sphere. **(A–F):** Arrows indicate clonally derived spheres and arrowheads indicate chimeric spheres. Scale bars = 200 $\mu$m.

microscope, we observed that the cells and spheres aggregated in the middle of the plate, resulting in significantly increased numbers of nonclonal spheres in each well (Fig. 2A–2C, $2A^1$–$2C^1$, stationary, vs. Fig. 2D–2F, $2D^1$–$2F^1$, moved). Since the dsRed and the YFP mice are on partially different background strains, we replicated the data at all experimental cell densities using two fluorescent mouse strains with similar backgrounds (YFP and mRFP mice) and found that the incidence of chimerism was similar to the present results (data not shown). Video time-lapse microscopy of primary sphere formation at 10 cells/$\mu$l without movement of the plates while being imaged indicated that the cells behaved as if they were in a stationary environment (i.e., left alone in an incubator), with only a 4% chimerism rate ($n$ = 246 spheres observed).

## Passaged Neurosphere Formation

Spheres from both the dsRed and YFP mouse strains harvested from neurosphere cultures that had undergone two or more passages (maximum, six passages), were dissociated and plated

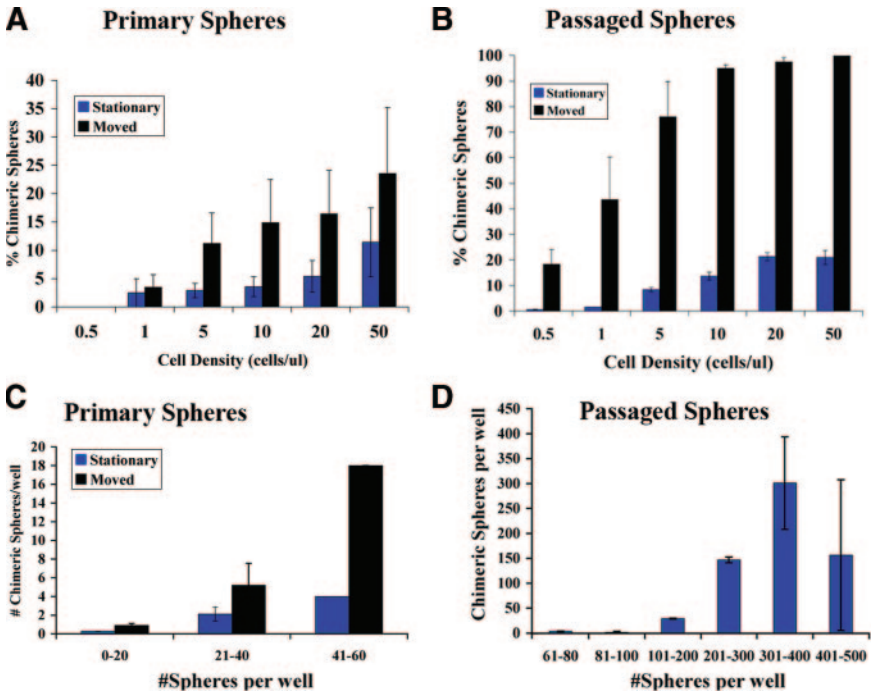


**Figure 3.** Clonality of neurospheres. **(A):** Primary neural stem cells formed clonal spheres at low densities in stationary wells (less than 5% of chimeric spheres at 20 cells/$\mu$l or less). However, if the plates were moved during the growth phase, there was a significantly higher incidence of chimerism. **(B):** Passaged spheres formed clonal spheres only at densities lower than 1 cell/$\mu$l (the incidence of chimerism was less than 5%); however, if the plates were moved, then even at 0.5 cells/$\mu$l many of the spheres were not clonal. **(C):** Graph of primary neurospheres grown in plates that were not moved, showing that the number of nonclonal spheres increased as the sphere density increased, which was exacerbated by movement. **(D):** Graph of stationary, passaged neurospheres demonstrating that most of the cell densities plated gave more than 100 spheres, and hence there was an increase in the incidence of chimeric spheres. The moved, passaged neurospheres aggregated into one large mass when plated at densities greater than 5 cells/$\mu$l; therefore, the data were not included in the graph.

at a 1:1 ratio at 0.5–50 cells/$\mu$l (Fig. 1A). There was no difference in the incidence of chimerism regardless of passage number; therefore, the passaged data from all passage numbers were combined. The cells proliferated over the course of 7 days, with one set of plates left stationary and the other set examined under the microscope daily, as was done in the primary sphere formation experiments. The incidence of nonclonal spheres from passaged cells was greatly increased at the low densities compared with the primary sphere formation. The plates that were left undisturbed during sphere formation were found to generate almost entirely clonal spheres only when cells were plated at less than 1 cell/$\mu$l (1.6% $\pm$ 0.02% [SEM] chimerism). At these low plating densities (0.5 and 1 cell/$\mu$l), the passaged cells gave rise to approximately 50–70 new spheres per well. However, the plates that were moved to the microscope daily were more than 10% chimeric at all of the cell densities tested, including 0.5 cells/$\mu$l (18.4% $\pm$ 5.7% [SEM] chimeric sphere incidence at 0.5 cells/$\mu$l) (Fig. 3B). The continuous video time-lapse microscopy of the passaged spheres at 10 cells/$\mu$l gave an overall 39% chimeric sphere rate ($n$ = 290 spheres).

## Sphere Density Affects Clonality

One of the issues with plating at a high cell density is that the estimation of the numbers of clonal spheres may be compromised by the greater number of spheres that arise (which result in an increased chance of the spheres fusing together). We expressed the data from both the primary and passaged sphere experiments above so that the number of chimeric spheres was plotted as a function of the total number of spheres that arose in each well. We arbitrarily divided the data at intervals of 20 spheres per well, and then at intervals of 100 spheres per well when there were more than 100 spheres per well. The chimerism of the spheres was positively correlated with the final number of spheres that arose in each well (Fig. 3C, 3D). There was a significant main effect of stationary versus moved plates ($F_{1, 10} = 15.4$; $p < .05$): the plates that were not moved had a lower incidence of chimerism. There was a significant main effect of sphere density ($F_{2, 10} = 10.9$; $p < .05$): the more spheres in the well, regardless of whether or not the well was moved, the greater the chimeric rate. There was also a significant interaction between movement and sphere density ($F_{2, 10} = 8.1$; $p < .05$): a greater number of spheres per well and moving the plates greatly increased the number of chimeric spheres compared with the same number of spheres per well in the stationary condition. In the moved plates, when the cell densities were 10 cells/$\mu$l or greater, the spheres tended to aggregate in the middle of the plate and formed one giant mass, which made it impossible to count the actual number of spheres present. The neural stem cells gave rise to primarily clonal spheres as long as there were fewer than 60 spheres per well in a 24-well plate (4% $\pm$ 0% [SEM] chimeric spheres). However, if the plates were moved during the growth phase, the maximum number of spheres that could be present in each well to ensure that the spheres were similarly mostly clonal was found to be 40 spheres per well (5.2 $\pm$ 2.4 [SEM] chimeric spheres). The finding that plate movement significantly interacts with sphere density (but that plate movement does not interact significantly with initial cell plating density; described in the initial section in Results) to increase the proportions of chimeric (nonclonal) spheres implies that the accumulation of fusing spheres increases as more spheres appear in the cultures.

## Encapsulation

To study stem cell behavior in a high-cell-density environment, a method was required that would allow the neural stem cell to remain clonal and proliferate to a sphere colony in the presence of large numbers of surrounding cells. We used an agarose encapsulation method [22] that keeps single cells from being able to enter or exit the capsule but allows factors in the surrounding serum-free media to freely diffuse through the agarose to the encapsulated cells (Fig. 1B). The encapsulated cells were derived from the passaged neural stem cells from the YFP mouse, and the unencapsulated cells were derived from the dsRed or CD1 passaged neural stem cells. We determined that single cells made up more than 98% of the capsules that contained cells. We found that the unapsulated single neural stem cells were capable of proliferating in the capsules and remained clonal as long as they were still encased in the capsule (Fig. 4). The cells were encapsulated within agarose capsules ranging in size from 50 $\mu$m to more than 200 $\mu$m. If the growing spheres reached the physical capacity of the agarose capsule,

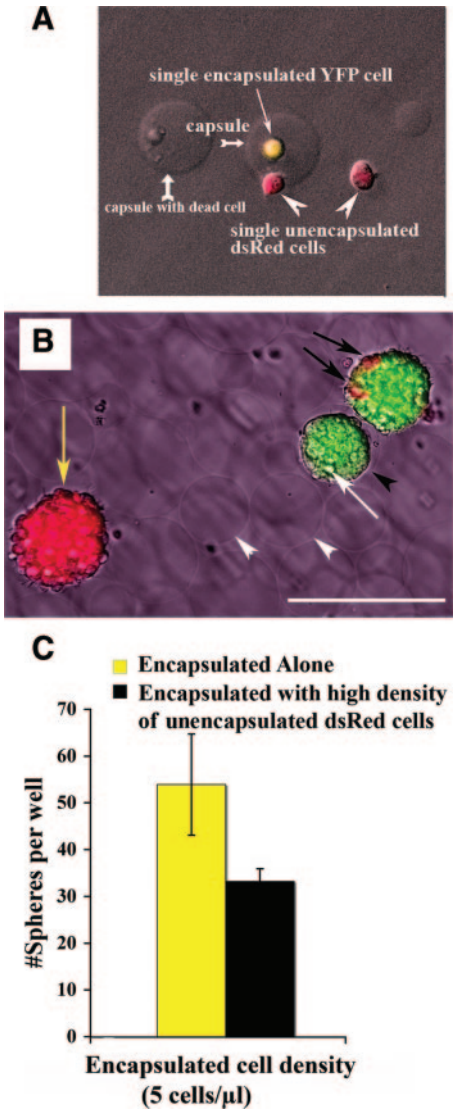


**Figure 4.** Encapsulation of stem cells. **(A):** Picture of a single encapsulated YFP cell with unencapsulated dsRed cells. The thin white arrow points to the YFP-positive cell inside the agarose capsule (indicated by the thick white arrow). The white arrowheads indicate the two unencapsulated dsRed cells. **(B):** A picture of separate encapsulated and unencapsulated neurospheres. The black arrowhead indicates a clonal encapsulated YFP neurosphere; the black arrow shows a few dsRed cells attached to the outside of a YFP nonclonal neurosphere, presumably after it burst out of the encapsulating gel; the yellow arrow points to a dsRed neurosphere that is most likely nonclonal due to the high plating; and the white arrowheads indicate a few of the many empty agarose capsules. The white arrow indicates a dead cell trapped in the agarose capsule that was not expressing any fluorescence. (Several other dead cells not indicated were also present in spheres.) Scale bar = 200 $\mu$m. **(C):** Graph of the numbers of clonal encapsulated EYFP spheres arising when plated alone at 5 cells/$\mu$l or when plated as encapsulated single cells (5 cells/$\mu$l) with a high density (100 cells/$\mu$l) of surrounding dsRed passaged neurosphere cells.

the spheres would burst out, and the unencapsulated cells and host spheres would readily attach to the newly "hatched" spheres (Fig. 4A). However, the spheres that were in capsules of more than 100 $\mu$m in diameter were still encapsulated 7 days after plating. The spheres were counted on day 4 (approximately 50 $\mu$m) to catch the proliferating spheres before many of them burst out of the capsules (approximately 100 $\mu$m). The encapsulated spheres could be single-sphere-passaged, giving rise to 30 or more new spheres per initial dissociated sphere and differentiated (each sphere giving rise to both neuronal and glial lineages) after being grown for 7 days in vitro, demonstrating self-renewal and multipotentiality (data not shown).

High densities (50 or 100 cells/$\mu$l) of surrounding unencapsulated dsRed cells, from dissociated dsRed neurospheres, were found to inhibit the number of single neural stem cells that proliferate clonally to form encapsulated spheres compared with the number of single encapsulated neural stem cells that proliferate to form spheres when plated alone without surrounding unencapsulated cells (with high densities [100 cells/$\mu$l] of unencapsulated cells: 33.3 $\pm$ 2.7 [SEM] clonal spheres; encapsulated single neural stem cells alone: 54 $\pm$ 10.8 [SEM] clonal spheres; $t_3 = 3.18$; $p < .05$) (Fig. 4B). Similar inhibitory results were found when CD1 passaged neural cells were used as the surrounding unencapsulated cell population to test a potential toxic effect of the dsRed cells (data not shown).

## DISCUSSION

There are three major findings here: (a) the neurosphere assay can give rise to clonal sphere colonies when neural stem cells are plated at low cell densities, (b) an experimenter can inadvertently cause chimerism in the proliferating spheres by moving plates and causing cell and sphere aggregation, and (c) when an encapsulation technique is used, single neural stem cells can give rise to sphere colonies, thus allowing the study of the effects of high densities of surrounding cells on the proliferation of single, clonal neural stem cell colonies.

The primary cell tissue isolated from the adult forebrain subependyma contains large numbers of postmitotic and progenitor cells, in addition to the rare population of highly proliferative neural stem cells; however, the passaged neurospheres contain a much higher percentage of stem cells per cell density plated. Therefore, the primary cell cultures could give rise to clonal spheres when plated at much higher plating densities; for example, more than 95% of the sphere colonies are clonal when primary subependymal cells are plated at less than 10 cells/$\mu$l (and not moved during the 7 days of culture) than passaged cells plated at the same density, because of the lower ratio of sphere-forming cells compared with non-sphere-forming cells (postmitotic or progenitor cells). A high percentage of the primary cells from dissociated subependymal lateral ventricular tissue undergo cell death within the first 24 hours of plating, which decreases the effective plating density [24]. This also means that a greater number of spheres will arise in a well that is plated from the passaged dissociated neurospheres, which in turn will increase the possibility of nonclonal sphere formation as the probability of the increased numbers of spheres colliding increases.

In all three studies [3–5] questioning the clonality of the neurosphere assay, the experimenters used passaged neurospheres. In the study by Jessberger et al. [4], the lowest density of passaged neurosphere cells plated was 5 cells/$\mu$l, which we have also demonstrated to be a nonclonal density for passaged neurospheres. Cells from passaged neurospheres were also used by Singec et al. [3]; in that study, cell densities as low as 0.5 cells/$\mu$l produced a chimeric neurosphere incidence of 40%, whereas we found a chimeric incidence of 5% at this cell density when the plates were not moved during sphere growth. Nevertheless, when the plates were moved in the present experiments, the nonclonal sphere incidence at a plating density of 0.5 cells/$\mu$l jumped up to 20%.

The present experiments indicate that experimental technique and observation can have an enormous impact on the clonality of neural stem cell colonies. It is important to remem-

ber that the neural cells and spheres are free-floating and therefore are vulnerable to any movement by the experimenter. We suggest that a Heisenberg-like effect (in which the observation affects the phenomenon under study [21]) is occurring in situations in which the plates are manipulated in any way during the neurosphere growth phase. In experiments in which the plates are moved to refeed the cells or to monitor the progress of the sphere formation under a microscope, there will be a higher incidence of nonclonal sphere formation. In the study by Mori et al. [5], the cells were refed every 3 days and time-lapse microscopy was performed with passaged cells. We have shown that moving the plates to the microscope repeatedly to check for sphere colony growth generates a fluid flow that aggregates spheres and induces chimerism (nonclonal sphere formation). It is probable that interference, such as moving the spheres to coated coverslips to quantify the clonality [3], will cause the fluid media to move and will also cause the cells and spheres to move closer together into the middle of the well, thereby leading to more nonclonal sphere colonies, much like aggregation culture experiments.

An alternative method of growing neural stem cell clones on a collagen substrate (NeuroCult; StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com) has been suggested as an easier and more accurate way to grow clonal neural stem cell cultures [6, 25, 26]. However, cells are plated a very low density in these collagen cultures (3 cells/$\mu$l in 35-mm dishes) for 21 days, whereas the neurosphere assay is clonal at 10 cells/$\mu$l or less and requires only 1 week to grow colonies (at least 100 $\mu$m in diameter) from single cells. To passage the NeuroCult-grown colonies, an enzymatic step and an intermediate plating step are required before the colonies can be dissociated into single cells, since the cells are very adherent to one another. The cells are mechanically dissociated into single cells and then plated, and in the process many of the cells are killed, resulting in the more difficult passaging of single colonies. In addition, the collagen matrix seemingly increases cell adhesiveness, since the neural stem cells that grow in the neurosphere assay require only a light mechanical trituration step to dissociate them into single cells. The neurosphere assay appears to be a more economical, less time-consuming, accurate method of isolating and prospectively identifying stem cells in vitro than the NeuroCult method. Indeed, neural stem cells from mice with a conditional knockout of $\beta$-catenin in the brain germinal zones will not form neurospheres, due to the loss of adhesion; however, in the NeuroCult semisolid media, they will form small clonal colonies that passage and are multipotential (manuscript submitted for publication), unlike small clonal wild-type neural stem colonies [6, 25, 26].Thus, the cell death during dissociation of small colonies from semisolid media may produce a serious underestimation of the numbers of neural stem cells.

Encapsulation of single neural stem cells in agarose ensured clonal growth of the sphere colonies. Plating cells at low densities or in single cell per well experiments can mask the potential of stem cells if they require certain factors that they cannot make themselves to survive or proliferate. In the hematopoietic system, it has been shown that there is a factor or factors keeping the stem cell quiescent both in vivo and in vitro and that removing progenitor cells away from the stem cells allows survival or expansion of the blood stem cells [27, 28]. The neural stem cell represents a very rare cell found in the subependymal region of the adult brain [29–31]. However, since it is impossible to prospectively and uniquely identify this neural stem cell in vivo, direct niche effects on the stem cell population are unable to be assessed. In principle, niche effects can be studied in vitro by observing the effects of high densities of host cells on single neural stem cells. Encapsulating single neural stem cells revealed that very high densities of neural stem cells and progenitors might actually have an inhibitory effect on the proliferation of single neural stem cells in culture. This may indicate that the large number of neural progenitor cells in neurosphere cultures may be releasing factors that are keeping the stem cells relatively quiescent in vitro. An alternative explanation for the decrease in proliferating neural stem cells may be that because of the high number of cells there is less proliferation, due to nutrient depletion; however, there was no difference in sphere diameter between encapsulated spheres at low densities versus the encapsulated cells grown in the high-density environment. The encapsulation technology will be useful for studying clonal stem cell behavior in environments with high cell densities of various types of surrounding cells or in experiments in which the cells will be subjected to any interference during the sphere growth stage, such as drug or media additions or movement for repeated microscopy.

## SUMMARY

In the absence of unique prospective markers, stem cells can be identified in vivo or in vitro only by first allowing the cells to proliferate clonally so that they then can be tested for self-renewal and multipotentiality (the hallmark features of stem cells). Thus, many stem cells can be identified only retrospectively when the cells are harvested and plated at very low cell densities or with single cells per well in vitro with nutrients and growth factors, and then allowed time to proliferate and form clonal colonies of cells. It must be noted that even in mixing two fluorescent mouse cell lines, we were effectively representing the mixing of dsRed with YFP cells and vice versa, but we were unable to identify dsRed mixing with dsRed and YFP mixing with YFP. Alternatively, single neural stem cells can be continuously monitored using video microscopy to ensure their clonal growth, as observed in the present and other [3, 4] experiments. However, single cell per well or continuous video imaging experiments can be time-consuming and costly. If cells are plated at a density and under conditions in which the clonality of the spheres that arise is ensured, then one can draw quantitative conclusions about the numbers of stem cells and assay their properties, such as self-renewal and multipotentiality, without having to resort to single cell per well experiments. Certainly, the studies that called the neurosphere assay into question did not use neural stem cells from primary brain tissue [3–5], which we have demonstrated are clonal at low densities. Moreover, it seems likely that experimenter-induced movement of plates, even at the very low cell densities of the passaged cells, produced aggregation (Heisenberg-like effects) in these past studies. The experiments in the present study have identified cell densities and conditions under which neural stem cells will proliferate to form clonal spheres, and they have identified the conditions under which the cells will give rise to nonclonal spheres.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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