Eyes Wide Open: A Critical Review of Sphere-Formation as an Assay For Stem Cells

Erika Pastrana[*,1], **Violeta Silva-Vargas**[*], and **Fiona Doetsch**

Departments of Pathology and Cell Biology, Neurology and Neuroscience, Columbia Stem Cell Initiative, Center for Motor Neuron Biology and Disease, Columbia University Medical Center, New York NY 10032, USA

Abstract

Sphere-forming assays have been widely used to retrospectively identify stem cells based on their reported capacity to evaluate self-renewal and differentiation at the single cell level in vitro. The discovery of markers that allow the prospective isolation of stem cells and their progeny from their in vivo niche allows the functional properties of purified populations to be defined. We provide an historical perspective of the evolution of the neurosphere assay, and highlight limitations in the use of sphere-forming assays, in the context of neurospheres. We discuss theoretical and technical considerations of experimental design and interpretation that surround the use of this assay with any tissue.

Stem cells are remarkable cells that are found in many tissues. They exhibit two cardinal properties: the ability to undergo self-renewal and the ability to differentiate. Because of these properties, stem cells are of crucial importance for maintaining tissue homeostasis and for tissue repair after injury. Great excitement has arisen about the therapeutic potential of stem cells, as well as recognition of their contribution to pathological states such as tumours. Changes in stem cell properties and the niches they inhabit may also have profound consequences for understanding aging.

To explore the dynamics, function and regulation of stem cells, and how these may go awry in disease, experimental assays must reliably be able to distinguish stem cells and their progeny. Due to the general lack of unique cell surface markers and the absence of a distinct and discernable morphological phenotype, stem cells have typically been defined and studied on the basis of functional criteria.

With the development of markers to prospectively identify putative stem cells, as well as sophisticated genetic approaches for lineage tracing, it is becoming increasingly feasible to define the dynamics of stem cells *in vivo*. Moreover, the ability to prospectively purify stem cells and their progeny has allowed their functional properties to be studied *in vitro* and their potential to be evaluated by transplantation *in vivo*. In the last few years, exciting discoveries have been made about the existence of quiescent and activated pools of stem

Corresponding Author: Fiona Doetsch, Columbia University Medical Center, 630 West 168th St, NYC NY 10032, USA, Tel: 212 342-5633, Fax: 212 305-5498, fkd2101@columbia.edu.
[*]Equal Contribution
[1]Present address: Nature Publishing Group, 75 Varick Street, New York NY 10013

cells and their ability to shuttle between these states. Transit amplifying progeny also have the potential to revert back to a stem cell state, at least in some tissues. As we discover more about the biology and behaviour of stem cells within their niches, and novel principles emerge, it is important to re-evaluate the strategies utilized to identify and functionally characterize adult stem cells. In particular, it is crucial to distinguish whether different paradigms evaluate actual *in vivo* stem cells or reveal stem cell potential, and to have a clear understanding of the strengths and limitations of different assays.

Stem cells from diverse tissues are typically cultured *in vitro* under non-adherent conditions as spheres, or under adherent conditions in two-dimensional cultures or in three-dimensional matrices. Sphere forming assays are widely used in stem cell biology, as theoretically both self-renewal and differentiation can be evaluated at the single cell level. In this protocol review, we critically assess the utility and the limitations of sphere-forming assays. As they were first used in the neural stem cell field almost twenty years ago, we provide an historical overview of the evolution of the neurosphere assay, which highlights important lessons that have been learned in the neural stem cell field regarding the identity of neurosphere-forming cells. Indeed not all neurospheres arise from stem cells, and this finding critically impacts the broadly held premise that sphere forming assays are a functional assay for uniquely detecting in vivo stem cells. Instead, sphere-forming assays evaluate the potential of a cell to behave as a stem cell when removed from its in vivo niche. We then outline additional important theoretical and technical considerations that incorporate emerging principles in stem cell biology that impact the interpretation of sphere-forming assays when used to evaluate stem cells from any organ.

The neurosphere assay: an historical perspective

The discovery of adult neural stem cells was the result of two coincident and divergent lines of research. The first was the re-investigation of adult neurogenesis and the second was the *in vitro* study of multipotent precursors from the adult brain.

Neural stem cells present in specialized niches in the adult mammalian brain continuously generate new neurons that are functionally integrated into neural circuits, including in humans. Adult neurogenesis occurs in two regions of the mammalian brain, the subventricular zone (SVZ), which is a thin layer of dividing cells adjacent to the lateral ventricles that generates olfactory bulb interneurons, and the subgranular zone (SGZ) in the hippocampal formation. These areas of continuous neurogenesis harbor stem cells that retain the capacity to proliferate, self-renew over an extended period of time, and differentiate into the three primary cell types of the brain (neurons, astrocytes and oligodendrocytes). As the neurosphere assay is almost exclusively used in the SVZ and not the SGZ, from which cells are predominantly cultured as adherent cells, the rest of this review is focused on the SVZ.

In the late 1960's, Joseph Altman first showed that new neurons are generated in the adult mammalian brain, yet this finding was largely ignored (Altman, 1969). In the 1980's the group of Fernando Nottebohm showed that new neurons functionally integrate into the adult songbird brain (reviewed in Nottebohm, 2004). However, it was not until the early 1990's, when new technical approaches were utilized, combining in vivo labeling and in vitro culture, that it was shown that precursors capable of giving rise to neurons were present in the adult mammalian brain and that neurogenesis and long distance migration occur *in vivo* (Lois and Alvarez-Buylla, 1993; Kirschenbaum and Goldman 1995; Lois and Alvarez-Buylla, 1994).

At the same time, Reynolds and Weiss first cultured cells that exhibit stem cell properties as free-floating spheres, called neurospheres, from the adult brain (Reynolds and Weiss, 1992). They dissected striatal tissue, which included the periventricular area encompassing the

SVZ, enzymatically dissociated the tissue to single cells and plated them in non-adherent conditions in serum-free medium in the presence of epidermal growth factor (EGF) (Figure 1). A small population of cells began to divide, initially adhering to the plate, and after a few days detaching and forming spheres of proliferating cells. The majority of cells within these neurospheres expressed nestin, an intermediate filament present in neuroepithelial stem cells in the embryonic brain. To assess whether cells could be propagated as secondary cultures, to show self-renewal, the neurospheres were mechanically dissociated and cultured again in the presence of EGF, with a subset forming secondary neurospheres (Figure 1). When plated on an adherent substrate, they differentiated into both neurons and glial cells. This provided the first evidence that multipotent stem cells were present in the adult mammalian brain.

Microdissection experiments subsequently revealed that the SVZ (also called the subependymal zone) is the source of neurospheres in vivo (Morshead et al., 1994). Moreover, it was also proposed that neurospheres only arise from relatively quiescent cells in vivo, based on a series of [3H]-thymidine kill experiments, in which neurospheres were cultured after the in vivo elimination of rapidly dividing cells (Morshead et al., 1994). However, as the cell types in the SVZ had not yet been defined, it was not clear which cells were present when neurospheres were cultured at different timepoints after the [3H]-thymidine kill. Further experiments revealed that neurospheres can be cultured from the entire ventricular axis of the central nervous system, including the spinal cord (Weiss et al., 1996; Vescovi et al., 1993). However, both bFGF and EGF are required for neurospheres to grow from these non-neurogenic brain regions. EGF neurospheres can only be cultured from the SVZ.

Since these early experiments, the neurosphere assay has evolved. It is now accepted that the assay needs to be performed at clonal density. To demonstrate multipotency, individual neurospheres must give rise to neurons, astrocytes and oligodendrocytes, the three main cell types in the brain, upon differentiation after withdrawal of growth factors. In addition, although at first neurospheres were considered to be a homogeneous population of nestin+ stem cells, it is now clear that individual neurospheres contain stem cells, progenitors and differentiated cells.

The neurosphere assay therefore appeared to provide a simple retrospective assay to identify cells exhibiting both functional properties of stem cells, self-renewal and differentiation, as well as a quantitative readout of the number of stem cells in vivo. Importantly, the identity of the in vivo stem cells had not yet been discovered and it was not feasible to prospectively isolate different cell populations from the SVZ to directly test which cells have the capacity to give rise to neurospheres.

Anatomy of the SVZ and identity of stem cells

A key step in identifying the stem cells responsible for adult neurogenesis, determining their in vivo lineage, and elucidating which cells give rise to neurospheres, was defining the cell types and architecture of the SVZ niche. The SVZ has several striking organizational features, best visualized in whole mount preparations that reveal the entire three-dimensional surface of the ventricular wall (Doetsch and Alvarez-Buylla, 1996). It has recently been shown that stem cells in the adult SVZ are regionally specified and that precursors for different interneuron subtypes reside in different regions (Hack et al., 2005; Merkle et al., 2007; Young et al., 2007; Kelsch et al., 2007; Ventura and Goldman, 2007). Newly generated neurons migrate from their sites of birth and collect in a network of migrating chains that extends throughout the SVZ to join the rostral migratory stream that leads to the olfactory bulb (Doetsch and Alvarez-Buylla, 1996). A small number of oligodendrocytes are also generated in the adult SVZ (Nait-Oumesmar et al., 1999; Menn et

al., 2006). However it is still unknown if tripotent stem cells exist *in vivo*, or whether separate stem cells give rise to oligodendrocytes and neurons.

Initially, ultrastructural analysis was used to identify the cellular populations in the SVZ, as there were no markers available to distinguish the different cell types (Doetsch et al., 1997). Indeed, two markers commonly used to identify neural stem cells, nestin and Sox2, are expressed by all cell types in this region, and therefore cannot be used as unique markers of stem cells (Doetsch et al., 1997; Tavazoie et al., 2008). Four main classes of cells are present in the SVZ (Figure 2). Multi-ciliated ependymal cells (Type E cells) line the ventricles. The chains of neuroblasts (Type A cells) travel through tunnels formed by the processes of glial fibrillary acidic protein (GFAP)+ cells with many ultrastructural features of astrocytes (Type B cells). Rapidly dividing transit amplifying cells (Type C cells) are clustered adjacent to the chains of neuroblasts.

Cell ablation and lineage tracing studies established the SVZ stem cell lineage. Intriguingly, cells with several hallmarks of glial cells, long thought to be support cells in the brain and derived from a completely different lineage than neurons, are stem cells in both adult neurogenic regions (reviewed in Kriegstein and Alvarez-Buylla, 2009). Within the SVZ, GFAP positive (Type B) cells are stem cells both during regeneration and under homeostasis (Doetsch et al., 1999a). They divide to generate transit amplifying cells, which in turn give rise to neuroblasts (Figure 2). Furthermore, a subset of GFAP+ cells form neurospheres. At the time that GFAP+ cells were identified as stem cells, ependymal cells were also proposed to be stem cells in the SVZ (Johansson et al., 1999). This debate was largely resolved as more sophisticated genetic labeling and purification strategies were developed. This finding was not replicated by others (reviewed in Kokovay et al., 2008), and the original group proposing that ependymal cells form neurospheres has recently published that they do not give rise to neurons under homeostasis (Carlen et al., 2009). However, several recent papers have resurrected the idea that multi-ciliated ependymal cells are stem cells, based on the claim that CD133 and FoxJ1 are exclusively expressed by ependymal cells and can be used to purify them (Coskun et al., 2008; Meletis et al., 2008). However, both markers are also expressed by a subpopulation of GFAP+ cells (see below); the specificity of these markers in non-neurogenic brain regions needs to be better defined using high resolution ultrastructural and molecular analysis. Later experiments in which dividing GFAP+ cells were killed in GFAP-TK mice confirmed that GFAP+ cells are the source of adult generated neurons (Garcia et al., 2004; Imura et al., 2003; Morshead et al., 2003). However, even now, a critical issue is the lack of markers available to distinguish between GFAP+ stem cells and other brain astrocytes, as well as how they differ in their functional roles and potential.

Another important, and ongoing issue, is the identity and dynamics of putative quiescent stem cells in the adult SVZ. Based on regeneration studies, a pool of slowly dividing B cells escapes being killed by an anti-mitotic drug and rapidly regenerates the SVZ (Doetsch et al., 1999a). Markers have not yet been defined that allow the purification of these cells. Moreover it is still unknown whether these cells are only activated during injury or whether a deeply quiescent pool participates in neurogenesis under homeostasis as well.

Relationship of prospectively purified cells to neurosphere-forming cells

A crucial advance in defining which cells form neurospheres was the identification of markers that allow populations at different stages of the lineage to be isolated or killed using genetic approaches.

Heterogeneity of GFAP+ Type B cells

A very active effort in the neural stem cell field is to define the heterogeneity of GFAP+ cells within the SVZ and elsewhere in the brain and assess their stem cell potential. Important insights have arisen from anatomical, ultrastructural and functional analysis. Early studies using electron microscopy identified two populations of GFAP+ Type B cells in the SVZ. Type B1 cells extend a process between ependymal cells to contact the lateral ventricle, and have a primary cilium (Doetsch et al., 1999b). Type B2 cells are located closer to the vasculature and divide more frequently (Doetsch et al, 1997) (Figure 2). Recent work has uncovered important new features of the SVZ niche (Mirzadeh et al., 2008; Shen et al., 2008; Tavazoie et al., 2008), which shed light on the functional organization of GFAP+ Type B cells. Ependymal cells are organized as a series of pinwheels along the ventricular wall with Type B1 astrocytes contacting the ventricle at the center of these pinwheels (Mirzadeh et al., 2008) (Figure 2). In addition, a planar vascular plexus extends throughout the length of the SVZ (Shen et al., 2008; Tavazoie et al., 2008). Both stem cells and transit amplifying cells are tightly associated with this vascular plexus and contact it at specialized sites that lack astrocyte endfeet (Shen et al., 2008; Tavazoie et al., 2008).

Several markers have now been identified that distinguish different subpopulations of Type B cells in the SVZ and have allowed their prospective purification (Pastrana et al., 2009; Beckervordersandforth et al., 2010). Importantly, some of these markers are co-expressed by multiple cell types, such as CD133 (prominin), which is expressed by both ependymal cells and a subset of B1 cells contacting the ventricle (Coskun et al., 2008; Mirzadeh et al., 2008, Beckervordersandforth et al., 2010). At least four populations of GFAP expressing B cells can be discerned within the SVZ: Type B1 cells contacting the ventricle (Doetsch et al., 1999b; Mirzadeh et al., 2008; Beckervordersandforth et al., 2010), some of which express CD133 (prominin); actively dividing (activated) EGFR+ astrocytes, some of which contact the lateral ventricle (Doetsch et al., 2002; Pastrana et al., 2009), and non-dividing multipolar niche astrocytes found closest to the striatum (García et al., 2004; Mirzadeh et al., 2008; Shen et al., 2008). The overlap of marker expression, proliferation state and morphology of these different subpopulations still needs to be elucidated.

Multiple populations give rise to neurospheres

Until recently, adult neural stem cells and their progeny have been difficult to purify using fluorescence activated cell sorting (FACS) due to the lack of markers that allow separation of cells at different stages in the lineage and direct comparison of their neurosphere-forming capacity. Diverse approaches have been used to attempt to isolate adult neural stem cells and their progeny, based on markers for cells at different stages in the lineage, cell cycle status, and putative general stem cell markers, including metabolic substrates, dye efflux, surface markers and fluorescently-complexed molecules and transgenic reporter mice (Table 1 and references therein). Table 1 summarizes the neurosphere formation efficiency of adult cells purified using different strategies. Interestingly, the side population purification method, in which Hoechst exclusion is used to prospectively isolate stem cells (reviewed in Golebiewska et al., 2011), is not selective for neurosphere-forming cells (Kim and Morshead, 2003). Similarly, aldehyde dehydrogenase activity does not significantly enrich for neurosphere-forming cells (Corti et al., 2006; Obermair et al., 2010).

Some combinations of markers have allowed significant enrichment of neurosphere forming cells from the adult SVZ (Table 1). However, most of the markers employed in these studies are common to several stages in the lineage and yield mixed populations of neural progenitor cells. When combinations of markers are used that allow the simultaneous isolation of different stages of the lineage, and the neurosphere forming capacity of each population assessed, it has become clear that neurospheres arise from cells within the

lineage that express EGFR and are in a proliferative state (activated GFAP+ stem cells and transit amplifying cells) (Table1; Doetsch et al., 2002; Pastrana et al., 2009). Consistent with these findings, killing of transit amplifying cells and dividing GFAP+ cells greatly reduces neurosphere formation (Doetsch et al., 2002; Garcia et al., 2004; Imura et al., 2003; Morshead et al., 2003). Both populations can be serially passaged and are multipotent. As such, the neurosphere assay does not provide an accurate readout of the number of stem cells in vivo. Moreover, the neurosphere assay likely does not detect quiescent stem cells, as the purified population containing putative quiescent stem cells does not give rise to neurospheres (Pastrana et al., 2009). The identification of markers that allow the isolation of quiescent stem cells will allow their sphere-forming capacity to be directly tested. Defining the populations of cells that form neurospheres after injury in various models will also be important to pinpoint potential latent stem cells elsewhere in the brain (Robel et al., 2011). It will be important to assess the long-term self-renewal capacity of different purified populations that can give rise to neurospheres both in vitro and after transplantation in vivo (Neumeister et al., 2009).

As revealed by this historical overview, the neurosphere assay cannot be used alone to define the in vivo stem cells. However, if performed carefully it can provide a useful tool to assay stem cell potential in vitro, in a relatively simple manner. Sphere-forming assays are increasingly used, both retrospectively and prospectively, to investigate stem cells and progenitors in many tissues during development and in the adult (Table 2), as well as in cancers and the cancer stem cell field (Hirschhaeuser et al., 2010; Clevers, 2011). Similarly, they are frequently employed to dissect the molecular regulation of self-renewal and differentiation, and to investigate how the intrinsic properties of stem cells/progenitors cells change with aging and pathology. For the appropriate interpretations of such experiments, it is essential to understand the strengths and limitations of this assay. Below we highlight critical considerations for sphere-forming assays that are relevant for all systems.

Critical Considerations for Sphere-Forming Assays

Over the years, experimental variability has been introduced into sphere-forming assays including medium composition and volume, cell density, surface area of the culture dish and duration in culture before quantification (reviewed by Chaichana, et al. 2006). This diversity in protocols has favored differing and sometimes conflicting results to arise from different groups. In Figure 3, we outline the steps and critical experimental parameters that are crucial in the design and execution of sphere assays. Below, we highlight general issues that are essential for the interpretation of sphere-forming assays (Table 3).

Cell density and clonality of spheres

Cell density is the most important and controversial parameter of sphere-forming assays as it has a critical impact on clonality. The final readout of sphere-forming assays is the size and number of spheres, whether primary or passaged. A central tenet of sphere-forming assays is that each sphere is derived from a single cell and is therefore clonal.

A wide variety of seeding cell densities, from presumably clonal to much higher cell concentrations, are used in different laboratories. Different groups consider a wide range of ratios of cells per volume of tissue culture medium to be consistent with a clonal density. Indeed, anywhere between 0.2 to 20 cells per μl is considered -or at least named- appropriate for clonal conditions of growth (Coles-Tabake et al., 2008, Ferrón et al.; 2007, Chojnacki and Weiss, 2008). Results obtained from high density seeded cultures are impossible to interpret due to fusion of spheres. Even low-density cultures can be problematic. Mixing experiments in which wild-type and fluorescently labeled cells are co-cultured suggest that a neurosphere can reliably be of clonal origin only when cells are plated at 10 cells/μl or 1

cell/µl when using primary cells and passaged spheres, respectively (Coles-Tabake et al., 2008). However, using imaging approaches, neurospheres were observed to frequently aggregate and fuse, even at low densities. Indeed, time-lapse imaging experiments show that free-floating neurospheres are highly dynamic structures, which undergo intrinsic, spontaneous locomotion (Singec et al., 2006; Mori et al., 2006), even if left untouched in the incubator. A second major cause of non-clonality is experimenter-induced aggregation (Coles-Tabake et al., 2008). Movement of plates to examine cultures under the microscope rapidly leads to the aggregation of spheres at the center of the plate. As such, true clonality can only be guaranteed by plating single cells per well.

Importantly, the choice of an appropriate cell density should be determined by the intended purpose of the individual sphere assay. If the experiment is designed to characterize and define the stem cell potential (self-renewal and differentiation) of a newly identified population in vitro, cells should be plated as single cells per well to ensure clonality. However, it is important to note that cell density directly impacts cell growth. Sphere-forming efficiency decreases significantly when cells are plated as single cells as compared to low-density conditions, due to autocrine/paracrine signals released by cells into the medium. If the purpose of the assay is to study other parameters, such as survival or proliferation, it may be possible to use low density cultures, as long as it is recognized that spheres may not be clonal. When spheres are passaged to assess self-renewal they should be re-plated at extremely low densities to avoid cell fusion and aggregation.

It is also important to perform clonal density cultures even at the primary sphere stage. Increasingly, primary spheres are cultured at very high density and then upon passaging plated at lower density. However, this practice can greatly impact the interpretation of results. To reiterate, both primary and passaged spheres should be cultured at clonal density. Finally, irregular clumps of cells resulting from cell aggregation can also appear in sphere cultures (Chen et al., 2005). For any stem cell system, it should be validated that spheres actually arise from proliferation (such as by a short pulse of a nucleoside analogue or by time-lapse imaging) and are not simply the result of the aggregation of cells.

To circumvent some of the above technical hurdles, variations of the classical sphere assay have recently been proposed. Encapsulating spheres or culturing them in semi-adherent conditions (i.e. methylcellulose or collagen), similar to colony forming assays in the hematopoietic field (Purton and Scadden, 2007), or microengineered hydrogel matrices may avoid problems associated with experimenter-induced aggregation and intrinsic mobility of spheres (Ignatova, et al., 2002; Cordey et al., 2008, Louis et al., 2008).

Sphere-forming assays may not detect quiescent stem cells

An important caveat of sphere forming assays is that they may not detect quiescent stem cells. Quiescent stem cells reside in a G0 state, which likely prevents their depletion in vivo and the possibility of the introduction of mutations during replication. In contrast, sphere-forming assays predominantly allow the expansion of cells that are either poised for proliferation *in vivo* or are already actively dividing, and can therefore be rapidly expanded *in vitro* with mitogens. As such, it may never be feasible to detect quiescent stem cells by sphere-forming assays, as the protocols used do not provide as yet unknown key components of the *in vivo* niche required for the activation of dormant stem cells, either during homeostasis or after injury. In addition, the intrinsic properties of quiescent stem cells may limit their rapid expansion in the presence of growth factors, such as inherently slower cell cycle kinetics, and an intrinsic limitation on the number of times they can divide before being exhausted. Once markers are identified that allow quiescent stem cells to be purified directly from their niche, insight will be gained into their physiology and molecular regulation.

Sphere-forming assays are not a read-out of in vivo stem cell frequency

Stem cell frequency is often calculated based on the number of spheres generated from a given tissue sample. This premise is based on the false assumption that all spheres are derived from a stem cell. Indeed, as described above, FACS purification of neural stem cells and their progeny have revealed that both stem cells and their transit amplifying progeny can give rise to neurospheres. Furthermore, this pattern has also been observed with spheres that arise from isolated mammary populations, termed mammospheres (Stingl, 2009). As such, sphere-forming assays are not a read-out of in vivo stem cell activity, but instead may reflect the potential of cells to exhibit stem cell traits.

Based on modeling of predicted and actual serial sphere forming capacity, it has been proposed that the neurosphere assay overestimates stem cell frequency by an order of magnitude (Reynolds and Rietze, 2005). Less than 6% of cells in neurospheres can be passaged more than seven times, suggesting that only a small fraction of cells exhibit extensive self-renewal (Louis et al., 2008). These findings were all based on retrospective analysis of neurosphere formation. Indeed, it has become common lore that serial passaging will eliminate more committed progenitors and select for self-renewing stem cells. However, both purified transit amplifying cells from the adult brain, which are short-lived cells in vivo, and multipotent progenitors in breast can be serially passaged and retain multipotency in vitro. Using prospectively purified populations, it will now be important to determine whether there is a difference in the number of times cells that were isolated at different stages can be serially passaged in vitro. While sphere culture conditions may allow long-term passaging of both cell types, this capacity might not be the case when cells are exposed to the in vivo niche. More challenging experimental conditions such as transplantation paradigms or in vivo lineage tracing are necessary complements of in vitro assays that will reveal differences in stem cell behaviour and potential.

Sphere size is not a read-out of in vivo stem cells

Significant heterogeneity exists in the size of individual spheres, independent of the problem of merging, and it has been posited that size indicates the nature of the founder clone. This premise is confounded by non-uniform criteria regarding what sized spheres to quantify, which typically ranges from 40–150 μm in diameter. Stem cells are believed to give rise to large spheres, and progenitors to smaller spheres. It has been postulated that only large spheres can be serially passaged, as opposed to smaller spheres, which cannot. However, this hypothesis has not been rigourously tested with prospectively purified cells. Indeed, the size of a sphere might also reflect responsiveness to growth factors as well as the proliferation/differentiation status of the parental clone-forming cell. This observation has important implications for interpreting sphere size in loss-of-function studies: smaller spheres could be a result of decreased self-renewal, or altered responsiveness to growth factors. Furthermore, the mode of division within a sphere can impact the size of a sphere. For example, smaller neurospheres (<100μm) grown in leukemia inhibitory factor (LIF) give rise to secondary neurospheres to the same extent as much larger neurospheres grown with EGF alone (Bauer, 2009). This fact is likely due to increased self-renewing divisions in the smaller spheres, and the presence of more differentiated cells in the large spheres, which are incapable of being passaged. Thus large clones may actually contain fewer stem cells than smaller clones.

Towards the prospective purification of sphere-forming cells

The ability to prospectively purify different populations of cells and assess their in vitro and in vivo behavior is a crucial advance in the stem cell field. Isolating stem and progenitor cells from solid tissues presents unique challenges to obtaining a viable single cell suspension. Stem cells from solid tissues are often relatively rare populations enmeshed in a

complex extracellular-rich microenvironment. As such it is crucial to optimize each isolation step to maximize yields (Figure 3). The use of FACS to isolate cells from solid tissue has recently been reviewed elsewhere (Alexander et al., 2009). Particular care has to be taken when FACS is used to isolate different populations, as both the enzyme used and duration of digestion can profoundly affect surface antigen survival, thereby influencing marker expression. Indeed, the expression patterns of CD133, CD15 and CD24 on embryonic neural progenitors are dramatically different depending on whether trypsin, papain, collagenase/ dispase or liberase 1 is utilized (Panchision et al., 2007), with distinct populations of cells appearing and disappearing depending on the enzyme. As more and more complex combinations of markers are used to FACS purify more refined populations of cells, this issue becomes increasingly important and might explain conflicting results observed by various groups that use different dissociation protocols. For example, it was recently reported that CD133+GFAP::GFP+ cells contain all neurosphere forming cells in the adult SVZ (Beckervordersandforth et al., 2010). However, when papain is used, instead of trypsin, both EGFR+GFAP::GFP+ and EGFR+ only cells give rise to neurospheres (Pastrana et al., 2009). As such it is crucial to ensure that the profile observed by FACS matches the in vivo expression pattern as well as to account for strain and species differences.

Another key issue is to validate the specificity of markers in vivo and to define the populations that express them. Sometimes it may be difficult to detect expression of a marker by rare cells if it is highly expressed by more abundant cells. An ongoing debate in the neural stem cell field is whether ependymal cells are stem cells in the SVZ (Johansson et al., 1999, Coskun et al., 2008). These claims are based on the putative selective expression of markers on ependymal cells. Both CD133 and FoxJ1 (Coskun et al., 2008; Meletis et al., 2008) have been suggested to be exclusively expressed by ependymal cells and been used to purify them, followed by neurosphere cultures. However, both markers are also expressed by non-ependymal, GFAP+ (Type B1) cells, which are highly enriched for neurosphere forming potential (Mirzadeh et al., 2008, Beckervordersandforth et al., 2010; Jacquet et al., 2009). This overlap highlights how important it is to establish the specificity of markers used to purify cells and assess their functional properties in vivo and in vitro.

A similar issue exists when labeling strategies are employed to prospectively identify cells based on their anatomical localization, for example the injection of lipophilic dyes, such as DiI, or of viruses encoding reporters, to label cells contacting a lumen. It is essential to ensure that the tracer is not transferred between cells, and that all of the populations that contact the lumen are known. In the brain, injection of tracers into the ventricles will lead to the labeling of both ependymal cells and GFAP+ stem cells that contact the ventricle (Figure 2C), which initially led to some confusion about the identify of neurosphere forming cells.

Markers are dynamic

Purified populations remain heterogeneous, and the iterative identification of additional markers will allow their further enrichment. However, an important point to consider is that two apparently distinct populations may actually be the same population of cells that are in different states or stages of the cell cycle. For example, the expression of Hes1, neurogenin2 and Delta1, proteins that are classically thought to distinguish various populations of cells in the developing brain, oscillates within the same cell in a cell cycle-dependent manner (Kageyama et al., 2010). CD133, a marker frequently used to isolate putative stem cells, is another excellent example of a protein that is influenced by the cell cycle state (Sun et al., 2009). In vivo, CD133 is expressed on primary cilia, yet in order to divide, cells must disassemble the primary cilium, as the centriole in the basal body is required for the centrosome. As such, these cells will lose CD133 expression. Finally, two other recent findings have important implications for the purification of different cells. An emerging theme in stem cell biology is that cells can shuttle between quiescent and activated states (Li

and Clevers, 2010) and that even more committed progenitors can revert back to a more primitive state (Davies and Fuller, 2008), underscored by the neutral drift that occurs in populations over times in diverse stem cell systems (López-García et al., 2010; Snippert et al., 2010).

Differentiation potential bias due to culture with exogenous growth factors

Traditionally, spheres are cultured in high levels of growth factors, in the presence of EGF (20 ng/ml), bFGF (10ng/ml) or both in many systems. Such high concentrations may bias the differentiation potential of the cultured cells. For example, standard culture conditions for neurospheres use high concentrations of EGF, which heavily biases the cells towards glial differentiation, both in vitro and after transplantation in vivo. Lowering the concentration of EGF promotes more neuronal differentiation (Burrows et al., 1997), but the neural stem cell field continues to use high levels of EGF in the medium. A second key point is whether the same or different cells grow in the presence of different growth factors. With the advent of the ability to purify different populations of cells by FACS, this issue can now be addressed directly. An important question is whether the multipotency observed in vitro also translates to the same set of fates being adopted in vivo, or whether this multipotent capacity is only unmasked in the specific conditions present in culture.

Alternatives to sphere-forming assays

Modifications to sphere-forming assays have been developed which circumvent some of the limitations described above. These include bioengineering approaches using patterned substrates to mimic the in vivo extracellular matrix and substrate elasticity, adherent two dimensional and three dimensional cultures, such as CFU assays and co-culture configurations with different niche components (reviewed in Vunjak-Novakovic and Scadden, 2011). With any of these newer variants, it is important to assess the behaviour of purified populations in each assay.

An increasingly widely used assay in the neural stem cell field is the neuronal colony-forming cell assay (NCFCA) (Louis et al., 2008), in which cells are cultured in a collagen-containing semi-solid matrix with EGF and/or bFGF. This assay retrospectively defines stem cells based on the size of the colony formed, with large colonies over 1–2mm in size postulated to be derived from stem cells, and all other smaller colonies from progenitors. This assay is based on the premise that progenitor cells exhibit limited proliferative capacity in relation to stem cells, and that the diameter of a colony can be used to distinguish its founder cell type. It will be crucial to directly test this assumption using purified populations of cells. While this assay circumvents the issue of aggregation, it still suffers from several of the same limitations of neurosphere assays, namely, dormant stem cells may not divide to form large colonies, colony size may simply reflect growth factor responsiveness, and the same biases of culturing in high levels of growth factors also remain.

Within a sphere, significant differentiation occurs due to complex cell-cell interactions. Two-dimensional adherent culture, in which stem cells and their progeny are expanded as monolayers, significantly reduces the number of differentiated cells during stem cell expansion. For example, culture of neural stem cells as cell lines as a monolayer of adherent cells in the presence of EGF and bFGF allows the propagation of a reasonably uniform population of cells with much less differentiation than observed in non-adherent assays (Conti et al., 2005; Pollard et al., 2009). However, this approach makes it more difficult to monitor single clones. Another promising approach is the co-culture of purified stem cells with different "niche" cells, in the absence of additional growth factors, which provide more physiological signals. Both endothelial cells and astrocyte co-cultures support the growth of neural stem cells (Lim and Alvarez-Buylla, 1999; Song et al., 2002; Shen et al., 2004;

Cheng et al., 2009). An attractive feature of 2D cultures is that morphological analysis and molecular characterization can easily be assessed. Moreover, time-lapse imaging of single colonies can reveal lineage dynamics of individual cells (Qian et al., 1998; Scheffler et al., 2005; Cohen et al., 2010; Costa et al., 2011).

Finally, three-dimensional Matrigel cultures have been very powerful in providing a proper microenvironment for clonal mini-organs to grow from single cells combined with other niche cells (Sato et al., 2009). Such three-dimensional cultures may constitute the ideal system to start assessing and manipulating quiescent stem cells in vitro that have not yet been able to grow in culture.

Towards the future

As our understanding of the in vivo biology of adult stem cells and their niche deepens, it is crucial to develop new in vitro assays that overcome the limitations and practical pitfalls of sphere-forming assays and their modifications highlighted in this review. These new assays will need to assess self-renewal and multipotency at a clonal level without biasing cells by introducing saturating levels of exogenous growth factors. Further development of technologies such as engineered culture matrices that allow single cell assays in both adherent and floating conditions will be an important step towards high throughput assays that can assess the role of different molecules on stem cell physiology. In combination with increasingly sophisticated purification methods these studies will enable the rigorous comparison of the biological differences between stem cells and their progeny. It will be important for the community to extend and standardize the use of these purification methods so that different assays can be cross-compared and their effect on the different populations better assessed. Importantly, stem cells cannot only be studied in isolation. In addition to the development of 3D culture models that recapitulate the in vivo niche it will remain essential to explore the biology of stem cell populations in vivo using transplant paradigms and in vivo lineage tracing of endogenous populations. Transplantation of purified stem cell populations or cultured cells back into their endogenous niches can complement in vitro assays in evaluating the in vivo potential of these populations. Moreover, serial transplantation studies could help determine the self-renewal capacities of these cells. Great leaps forward will continue to be made by the synergy between in vivo and in vitro approaches, which mutually inform each other.

Acknowledgments

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Figure 1. Schema of the neurosphere assay

Figure 2. SVZ anatomy and lineage
A, Frontal schema of adult mouse brain showing SVZ in orange adjacent to the lateral ventricle.
B, Summary schema of the organization of SVZ cells. GFAP+ stem cells (B, blue) transit amplifying cells (C, green), neuroblasts (A cells, red), are adjacent to ependymal cells (E, grey), which line the lateral ventricle (LV). A subset of GFAP+ cells (B1) extend a process between ependymal cells to contact the LV. Stem cells and transit amplifying cells directly contact the vasculature at specialized regions on blood vessels lacking astrocyte endfeet (dark blue) and pericyte coverage (yellow).
C, Confocal image of ependymal cell pinwheel with GFAP::GFP+ Type B1 cell contacting the ventricle at its center (Image from Angel Maldonado-Soto, generated according to the methods reported in Mirzadeh et al., 2008).
D, Stem cell lineage and sagittal schema of adult mouse brain showing whole mount perspective of SVZ adjacent to lateral ventricle (red). Newly generated neurons migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB). Small numbers of oligodendrocytes are also generated in the SVZ but are not shown here. Beige boc indicates neurosphere forming cells in the lineage.

Tissue Digestion

Microdissection → Enzymatic Digestion → Clean-up Step

Microdissection
•Microdissection increases the yield of sphere-forming populations

Enzymatic digestion
•Enzymatic digestion allows isolation of specific cell types and avoids cell death induced by mechanical digestion.
•The particular enzyme used, length of digestion and amount of tissue alter cell viability, dissociation efficiency and surface marker profiles.

Clean-up step
•Debris clean up, such as Percoll step: Important to ensure cell of interest not lost.

Bulk   **FACS**

Validate marker expression → Cell viability → Event number and purity of cells

Validate marker expression
•Markers are dynamic and may be expressed by multiple cell types. Critical to define which populations express marker of interest in vivo.
•Verify promoter specificity and correlation between fluorescence and endogenous protein and RNA.
•Ensure that the digestion protocol does not alter expression of surface antigen.

Cell viability
•Essential to exclude dead cells to increase viability
•As assay is performed in serum-free medium, serum should be avoided at all steps in protocol (including immunostaining) as transient exposure to serum can affect cell behavior. Serum is often used in collection tubes to increase survival of cells. BSA can be used as alternative for staining and defined supplements for collection. Need protein in collection medium to provide a "cushion" to avoid significant loss of cells during sorting.

Event number and purity of cells
•Perform acute immunostaining to assess heterogeneity of purified population
•Event number does not accurately reflect cell number. It is critical to define actual plating efficiency after 1 day for accurate quantification of sphere formation

Plating Cells

Cell density → Single Cell / Low density → Mitogen combination

Cell density
•Cell density is a critical parameter as it will influence clonality. It is essential to plate known number of cells.
•Cells aggregate if cell density is too high and due to experimenter induced movement of dishes
•Sphere forming efficiency is influenced by autocrine/paracrine signals; as such, different cell densities affect sphere numbers
•Account for sphere-forming capacity of population as factor in plating density.
•Single cell plating is recommended to assay clonal stem cell properties in vitro.
•Determine plating efficiency and viability after plating
•Ensure spheres are due to proliferation and not aggregation

Mitogen combination
•Choice of mitogen can influence proliferation and fate potential of cells.

Quantification

Quantification
•Quantify at consistent time after plating
•Record sphere size

Passaging and differentiation

Passaging
•Important to obtain single cell suspension without killing cells and plate at clonal density plating to avoid aggregation

Differentiation
•Choice of substrate and medium/growth factors can influence differentiation

Reporting

Reporting
•Number of cells per ul should be reported
•Represent neurosphere efficiency as number of neurospheres/cells plated
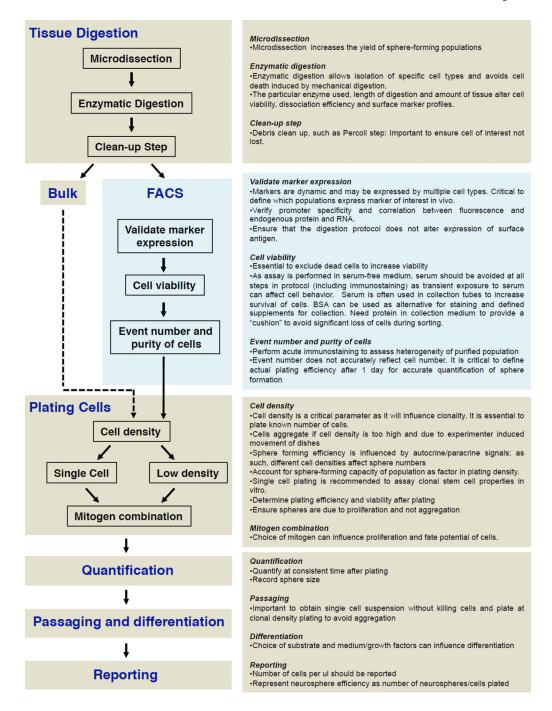
Figure 3.
Flowchart outlining design and critical experimental steps in sphere-forming assays

Table 1

Markers used to purify neurospheres from the adult brain and their reported efficiencies

Marker	Isolation Strategy	Reported % NS formation from adult	Reference
Bulk frequency	Bulk dissociated cells	0.03%--1%	
Aldehyde dehydrogenase	ALDH activity with aldefluor substrate	2.82%	Corti et al 2006
Cell size/PNA/CD24	Cells > 12μm/ anti-PNAlow cells/ anti-CD24low	80%	Rietze et al 2001
CD15 (Lex, SSEA-1) or CD24	Anti-CD15 or Anti-CD24	CD15+ 25% CD24+ 0.15%	Capela and Temple 2002
CD133 (Prominin)	Anti-CD133	4%	Corti et al 2007,
CD133 or CD24	Anti CD133 or Anti-CD24	CD133+ 6% CD24+ 0.9%	Coskun et al 2008
CD133/CD24	Anti CD133/Anti-CD24	CD133+/CD24+ 0%	Pfenninger et al.2011
CD133/CD15/aldehye dehydrogenase	anti-CD133/anti-CD15/aldehyde dehydrogenase activity with aldefluor substrate	CD15+ 0.45% Aldh high 0.41% Aldh high/CD15+ 0.24% did not detect CD133+/CD15+ cells	Obermair et al 2010
Dcx	Dcx::GFP	Dcx Low 0.8% Dcx- 1.2%	Walker et al 2007
Dlx2/CD24	Dlx2::LacZ/Anti-CD24	Dlx2+CD24– 14%	Doetsch et al 2002
Dil labeling (Intraventricular injection)	Intraventricular injection of DiI	Dil+ 6.2%	Johansson et al.1999
Erythroagglutinin lectin (E-PHA)	FITC conjugated E-PHA	Low E-PHA 0.02% High E-PHA 0.26%	Hamanoue et al 2009
FGF1B	F1B::GFP	1%	Hsu et al 2009
GFAP	GFAP-GFP adenoviral labelling	11.65%	Doetsch,et al.,1999b.
GFAP/EGFR/CD24	hGFAP::GFP/Fluorescent EGF ligand/anti-CD24	GFAP+EGFR+CD24– 30% EGFR+ 7.5% CD24low 0% GFP+ 0%	Pastrana et al 2009
GFAP/CD133	hGFAP::GFP/antiCD133	78%	Beckervordersandforth et al 2010
Id1 reporter activity	Id1::GFP	Id1GFP high 1% Id1GFP low 0.5%	Nam and Benezra 2009
Mcm2	Mcm2::GFP	3%	Maslov et al 2007
Nestin	Nestin::GFP	0.30%	Kawahuchi et al 2001
Notch 1	Anti-Notch1	Notch1+ 2.8%	Johansson et al.1999
Notch reporter activity	TNR::EGFP	EGFP ^{high}70% EGFP ^{low}10%	Andreu-Agulló et al 2009
Side population (SP)	exclusion of Hoechst 33342	SP 2.1% Non-SP 0.28%	Kim and Morshead 2003
Sox1	Sox1::GFP	1.70%	Barraud et al 2005
Sox2/beta1 integrin	sox2::GFP/ beta1 integrin	Sox2GFP+beta1+ 0.05% Sox2GFP +beta1– 47%	Kazanis et al 2010
Selective killing experiments			

Marker	Isolation Strategy	Reported % NS formation from adult	Reference
DLX2	Dlx2-TK	70% of ns are killed	Doetsch et al 2002
GFAP	GFAP-TK	~100 % of ns are killed	Imura et al., 2003; Morshead et al., 2003; Garcia et al., 2004

Table 2

Tissues in which sphere-forming assays have been used

Tissue	Isolation markers	References
Breast	- Human bulk breast tissue - Lin (CD45/CD31/Ter119)/CD29/CD24	Dontu et al., 2003 Shackleton et al., 2006
Cornea	Bulk microdissected human corneal epithelium	Yokoo et al., 2005
Dermis	Bulk skin tissue	Toma et al., 2001
Heart	Side Population	Tomita et al., 2005
Pancreas	- Bulk microdissected pancreatic islets and ductal tissue - ALDH1/ E-cadherin	Seaberg et al., 2004 Rovira, et al., 2010
Pituitary gland	Side Population	Chen et al., 2005
Prostate	Lin (CD45/CD31/Ter119)/Sca1/CD49f	Lawson et al., 2007
Retina	Bulk microdissected ciliary margin of outer retinal pigmented epithelium	Tropepe et al., 2000
Trachea	- KRT5-GFP - Human bronchi: Itga6/NGFR	Rock et al., 2009

Table 3

Overview of critical considerations for sphere-forming assays

Cell density and clonality of spheres	Cell density is critical parameter as it influences clonality. Spheres are prone to aggregation due to both intrinsic locomotion and to experimenter-induced movement. Clonality is only guaranteed by single cell plating. Important to ensure that spheres are due to proliferation of cells and not to aggregation.
Sphere-forming assays may not detect quiescent stem cells	Sphere forming assays predominantly detect cells that are poised for, or are actively undergoing, proliferation. Quiescent cells may not be capable of forming spheres, either due to intrinsic cell properties or due to lack of additional extrinsic signals needed for their activation in this assay.
Sphere-forming assays are not a readout of in vivo stem cell frequency	Multiple populations in stem cell lineages, including both stem cells and transit amplifying cells, are able to form spheres that can be serially passaged and are multipotent. The long-term in vitro and in vivo potential of purified populations needs to be assessed.
Sphere size is not a read-out of in vivo stem cells	Large spheres are often assumed to arise from stem cells. However, independent of aggregation issues, sphere size simply reflects proliferation/ differentiation status and responsiveness to growth factors of the parental clone-forming cell.
Towards the prospective purification of sphere-forming cells	As FACS becomes an integral part of assaying the potential of different populations to form spheres, it is essential to ensure that enzymatic digestion does not alter surface marker profiles (different enzymes result in markedly different surface profiles). In addition, the cell type specificity of individual markers needs to be validated in vivo.
Markers are dynamic	Purified populations reflect the state of a population at a given moment in time. Within a purified population, cells may be in different states or stages of the cell cycle. Markers commonly used to purify stem cells change their expression with the cell cycle. Cells can shuttle between quiescent and activated states, or from more committed to more primitive states.
Differentiation potential bias due to culture with exogenous growth factors	Cells within a population may respond differently to distinct mitogens. Mitogens can also bias the differentiation capacity of cells.