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SPOTLIGHT

In vivo imaging: shining a light on stem cells in the living animal

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ABSTRACT

Stem cells are undifferentiated cells that play crucial roles during development, growth and regeneration. Traditionally, these cells have been primarily characterised by histology, cell sorting, cell culture and *ex vivo* methods. However, as stem cells interact in a complex environment within specific tissue niches, there has been increasing interest in examining their *in vivo* behaviours, particularly in response to injury. Advances in imaging technologies and genetic tools have converged to enable unprecedented access to the endogenous stem cell niche. In this Spotlight article, we highlight how *in vivo* imaging can probe a range of biological processes that relate to stem cell activity, behaviour and control.

KEY WORDS: *In vivo* imaging, Stem cell, Cell behaviour

Introduction

Stem cells are defined as long-lived cellular populations that possess the unique properties of self-renewal and the ability to provide a source of differentiated cells for organ growth, homeostasis and regeneration. In recent years, there has been intense interest in characterising how stem cells undergo self-renewal, how they interact with each other and the local tissue microenvironment, and how they respond to different cues to regulate cellular growth and differentiation. Classic histological and *ex vivo* cell culture methods have allowed the molecular characterisation of stem cells isolated from different tissues. These studies have provided substantial insights into stem cell function, but have also led to competing models of the mode of stem cell action. A major limitation has been an inadequate ability to test the validity of these models by visualising stem cell behaviours in real time within intact organ systems *in vivo*. Imaging tools that allow the direct visualisation of stem cells within their native environment are extremely valuable in studying dynamic stem cell behaviour; however, there are still a number of challenges that limit the widespread use of *in vivo* imaging during stem cell activation. In this Spotlight, we discuss these challenges, potential solutions, as well as examples of unexpected behaviours that have been uncovered by examining the activity of stem cells within living tissue.

Visualising stem cells in their native environment: insights from different animal models

Most mammalian stem cell niches reside deep within their resident tissue and therefore, until recently, imaging techniques have been inadequate to probe the cellular dynamics of these cells. Furthermore, mammalian tissues are often opaque to the wavelengths of light commonly used during light-based imaging, further limiting the capacity to document cellular behaviours. Despite these limitations, researchers have persisted in experimenting with *in vivo* stem cell imaging in mammalian systems and several recent studies are testament to their success (discussed below and reviewed by Park et al., 2016).

Given the difficulty in accessing tissue-resident stem cells in larger animals, studies on invertebrate species have dominated our understanding of the molecular and cellular processes that control *in vivo* stem cell behaviours. Invertebrates such as fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*) have been used to visualise *in vivo* cell dynamics due to their small size, tissue accessibility and ease of transgenic approaches (Wong et al., 2013; Rosu and Cohen-Fix, 2017). Owing to their genetic tractability and the relative ease of cell-specific genetic manipulation, *Drosophila*, in particular, has revealed novel insights into the molecular interactions between cells. Indeed, the germ cell niche has been intensively studied and has revealed specific stem cell self-renewal behaviours that regulate cell numbers during development and regeneration (Fichelson et al., 2009; Morris and Spradling, 2011; Sheng and Matunis, 2011). However, even in this model the study of stem cells in most adult organs remains challenging.

In recent times the most widely adopted vertebrate model system in which to examine *in vivo* dynamics of various stem cell populations is the zebrafish. This is primarily due to the optical clarity of both embryos and larvae, which enables deep tissue visualisation, as well as the comparative ease in zebrafish of generating genetic manipulation tools to label specific cell types. The power of *in vivo* cell imaging in zebrafish is illustrated by its use to gain a deeper understanding of how stem cells and their progeny are maintained and interact with each other within tissues that are usually inaccessible in mammals, such as the brain (Barbosa et al., 2015; Dray et al., 2015), hair cells (Pinto-Teixeira et al., 2013), retina (Das et al., 2003; MacDonald et al., 2015), haematopoietic tissue (Bertrand et al., 2010) and skeletal muscle (Gurevich et al., 2016; Nguyen et al., 2017).

Using these model invertebrate and vertebrate organisms, the dynamic nature of stem cells has been visualised at considerable resolution within their distinct *in vivo* niches. Similar analyses within mammalian tissues have required considerable technical advances in microscopy and, in general, these studies have lacked the temporal and spatial resolution available in other systems. However, recent approaches have begun to bridge this gap, and collectively these studies have begun to define conserved stem cell behaviours within and between homologous organ systems.

Improvements in microscopy systems

Since most stem cells cannot be distinguished using regular light microscopy, fluorescence microscopy must be employed, making use of transgenic animals fluorescently labelled for the cells of interest. The confocal microscope is now so widely accessible to most research laboratories that it has become the default instrument in live imaging studies.

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Despite the relatively inexpensive and simple set-up for confocal microscopy, this method of imaging is still constrained by the limited capacity of the microscope to image deeper tissues [greater than ∼30 µm (Centonze and White, 1998)], as light scattering limits imaging resolution. Furthermore, since the tissue above and below the plane of focus will also be exposed to the excitation light, the problem of photobleaching and phototoxicity becomes more pronounced when cells of interest are located very deep within the tissue. The recent advent of light sheet fluorescence microscopy (LSFM) techniques such as single plane illumination microscopy (SPIM) (Huisken et al., 2004) and lattice light sheet microscopy (LLSM) (Chen et al., 2014) has greatly lessened the impact of photobleaching and phototoxicity, as they employ planes of light instead of a point source to scan samples. This provides a more rapid method of data capture than conventional confocal microscopy, with the added bonus that only the section of the sample under observation is illuminated.

Although these techniques have been rapidly adopted in optically accessible animal model systems, primarily by invertebrate and zebrafish researchers, the use of LSFM is limited to the imaging of transparent samples. Furthermore, as in standard confocal microscopy, the quality of imaging deteriorates with focal depth (a problem most acute in LLSM), thus making LSFM-based techniques unsuitable for *in vivo* imaging of stem cells resident deep with opaque tissues, a situation that most often confronts mammalian stem cell biologists.

The development of multiphoton microscopy has at least partially addressed some of these issues (Helmchen and Denk, 2005). This imaging modality is based on the ability to excite a fluorophore by simultaneously exposing it to two low-energy photons. Since the fluorophore will only be excited where the two photons meet, the fluorophore can be targeted at a focal plane and the surrounding tissues will not be exposed to the excitation light. Therefore, cells deep in tissues [up to ∼60-80 µm (Centonze and White, 1998)] can be imaged with reduced photobleaching, phototoxicity and autofluorescence. Multiphoton imaging systems can also make use of images generated through second harmonic wavelength production, which occurs when two photons of one wavelength are absorbed and a single photon of half the wavelength is consequently emitted. This is primarily used to visualise collagen networks *in vivo* and can consequently provide information on tissue and niche architecture without the need for additional fluorophore labels. The development of this imaging system has allowed, for the first time, the *in vivo* visualisation of mammalian stem cell populations, in particular in the hair follicle (Rompolas et al., 2012, 2016) and more recently the epidermis (Mesa et al., 2017 preprint).

Multiphoton microscopy has underpinned the development of intravital imaging, which allows the visualisation of cellular dynamics within various mammalian tissues in the living animal using the implantation of imaging windows and devices. The dynamics of a number of stem cell systems of adult mice have been studied within intact organ systems using intravital imaging including, most recently, the adult mouse brain (Pilz et al., 2018). We provide three examples among many to illustrate the power of the approach to resolve long-standing questions in the field.

Using an imaging window placed in the skull of an adult mouse, the haematopoietic stem and progenitor cells within the calvarial bone marrow were shown to interact in a specific and highly regulated manner with blood vessels, osteoblasts and endosteal surfaces as they homed and engrafted in irradiated transplanted mice, processes that had not been documented previously (Lo Celso et al., 2009).

Another well-studied stem cell paradigm is the mammalian intestinal epithelium. The rapid turnover of this tissue is facilitated by stem cells located at the base of the intestinal crypt. Previous studies using retrospective clonal analyses had established the paradigm of neutral competition between dividing stem cells to generate dominant clones during cell replacement (Snippert et al., 2010). However, how the heterogeneous intestinal stem cell pool was regulated initially to establish a dominant stem cell clone was not at all clear from these results. Using intravital imaging via a surgically implanted abdominal imaging window in adult mice transgenically marked for the intestinal stem cell niche, it was observed that certain stem cells had a preference to fully colonise the crypt, which was determined by the proximity to the niche itself (Ritsma et al., 2014). This result explained how a heterogeneous stem cell population functions to establish long-term self-renewal potential (Fig. 1A) (Ritsma et al., 2014).

The nature of the stem cell hierarchies in the epidermis of the mouse, another tissue with rapid cellular turnover, has been highly debated for a number of decades. Uniquely amenable to 'non-invasive' two-photon imaging, the analysis of individually tracked

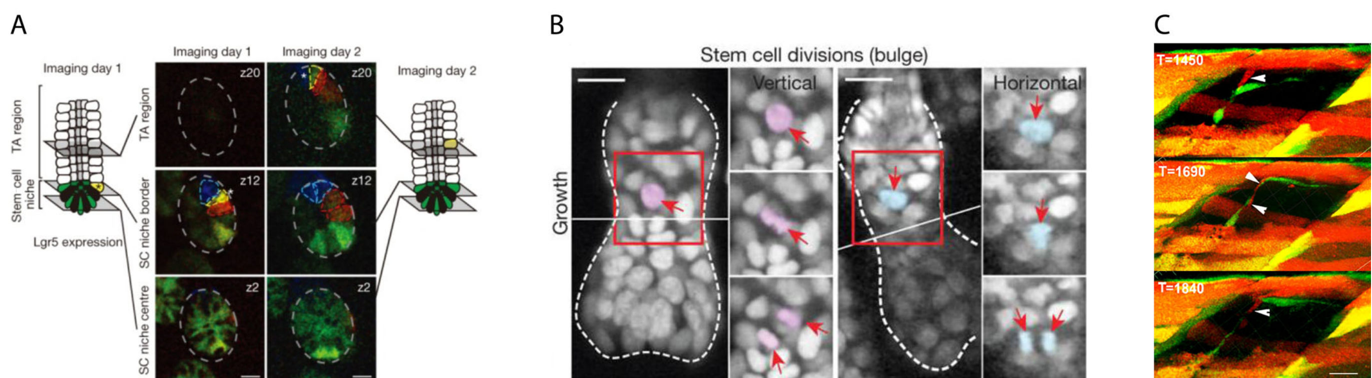

Fig. 1. Examples of imaging live cell dynamics *in vivo*. (A) In the mouse intestinal stem cell system, multicolour labelling of individual clones via the Confetti mouse followed by continuous imaging reveals the movements of clones outside of the stem cell niche – the yellow cell (asterisk) moving from the stem cell (SC) niche to the transient amplifying (TA) region. Adapted with permission from Ritsma et al. (2014). (B) In the mouse hair follicle stem cell system, imaging of individual cells (arrowed) during the growth phase within the stem cell niche reveals orientated cell divisions according to the direction of the hair follicle. Adapted with permission from Rompolas et al. (2012). (C) In the zebrafish muscle stem cell system, an uninjured differentiated muscle fibre (red fibre, arrowhead) extends to 'grab' the regeneration-specific stem cell (green) and 'guide' it into position along the pre-existing fibres. T is time after injury (minutes). Adapted from Gurevich et al. (2016). Scale bars: 20 µm.

and marked cells within the epidermis has recently revealed that epidermal stem cells have equal potential to either divide or directly differentiate, a process seemingly coordinated by the fate of sibling cells (Rompolas et al., 2016). These observations have led to a model of epidermal stem cell function that is distinct from those previously postulated (Rompolas et al., 2016).

Using *in vivo* imaging to probe the dynamics of organ growth and regeneration

Tissue regeneration is a prime example of a cellular process where *in vitro* and *ex vivo* models struggle to mimic bona fide *in vivo* complexity, which involves multiple cell types and factors that influence stem cell proliferation and differentiation during repair. A number of studies in both zebrafish and mice are beginning to probe the dynamics of tissue regeneration through sophisticated live imaging approaches. For example, hair regrowth (anagen) in mice is a natural regenerative process initiated by specialised mesenchymal fibroblasts or dermal papilla, which induce proliferation in the juxtaposing epithelial stem cells of the hair follicle. Recent two-photon imaging studies have revealed how this complex process is orchestrated at the cellular level, documenting a highly dynamic and compartmentalised mechanism of stem cell activation. The end result is a pool of stem cells that is sufficient for both cellular replacement and hair cell regeneration. The cellular interplay between these processes would not be revealed by the examination of individual stem cells in isolation (Rompolas et al., 2012, 2016) (Fig. 1B). Intravital imaging of mouse skin epithelium has also been used to characterise the dynamics of wound repair. Although this specific study did not resolve individual stem cell behaviours, global cellular properties such as directed division and migration behaviours were described, revealing how wound repair can occur while maintaining healthy tissue homeostasis (Park et al., 2017). The goal for studying stem cell dynamics in epidermal wound healing will now be to achieve the level of resolution documented in the process of hair cell regeneration.

In contrast to the epidermis, mammalian skeletal muscle has proved to be a difficult tissue in which to image the dynamics of tissue regeneration. Skeletal muscle harbours a well documented and relatively simple stem cell repair system, the satellite cell, which acts to repair damaged muscle fibres after injury and during disease. Despite its simplicity, visualising the activity of the stem cell niche *in vivo* has been extremely challenging and the cues that coordinate muscle stem cell behaviour remain poorly described in the *in vivo* context. Recent studies using an imaging window positioned on the mouse tibialis anterior muscle revealed how the extracellular matrix of damaged muscle fibres directs specific muscle stem cell divisions. This unexpected result highlights the important role of the wound site itself in orchestrating repair, rather than the fates of satellite cell division being an intrinsic property of the activated stem cell (Webster et al., 2016).

Studies in zebrafish have produced complementary results that also illustrate the importance of the wound site in orchestrating stem cell activity during muscle repair. However, an additional discovery in this model system revealed that an asymmetric division of the stem cell generates a regeneration-specific population (Gurevich et al., 2016). This model was predicted by *ex vivo* and *in vitro* observations (Conboy and Rando, 2002; Shinin et al., 2006; Kuang et al., 2007; Rocheteau et al., 2012; Troy et al., 2012), but the *in vivo* study also uncovered a series of highly complex and unexpected cellular behaviours at the wound site. Perhaps most unexpected of these observations was the extent to which the uninjured muscle fibres neighbouring the wound site orchestrate the regenerative process, guiding stem cells towards the injury area to align them with pre-existing fibres (Gurevich et al., 2016) (Fig. 1C). Revealing such unique and dynamic cell behaviours was only made possible through continuous live imaging. It will be intriguing to examine how other stem cells within distinct tissues are directed by the wound site to effect repair.

In a given stem cell population, the decision to either expand or provide cells for differentiation must be highly regulated. This homeostatic control paradigm is necessarily modified during growth, as the stem cell niche must accommodate the large increase in cell numbers required to allow the expansion in the size of an embryo into a fully formed adult, but how this is controlled is largely unknown. Recent studies in zebrafish skeletal muscle have revealed that a growth-specific, low-cycling stem cell population generates a clonal pool of progenitors that are responsible for the formation of the entire adult muscle (Nguyen et al., 2017). This process of clonal drift during skeletal muscle growth is consistent with a recent study using a CRISPR/Cas9-based lineage tracking technique that demonstrated that adult organs derive from relatively few progenitors (McKenna et al., 2016). These studies collectively suggest that clonal drift might be a universal property of stem cells within all organs, a hypothesis supported by analyses of stem cell dynamics across multiple systems (Klein and Simons, 2011). With the generation of novel lineage-tracing tools and imaging modalities, it will be exciting to determine whether this model is indeed generally applicable to the majority of growth stem cell niches.

Concluding remarks

In vivo imaging has provided unique insights into how stem cells behave in their niche and respond to different biological cues. There is now increasing evidence that stem cells, rather than being static and linear in their proliferative and self-renewal capabilities, are instead highly dynamic, interacting with distinct niche cells, an ever changing extracellular environment and each other and their progeny, to regulate function. A significant immediate goal, enabled by the increasing capacity and resolution of emerging imaging modalities, will be to deliver high-resolution imaging of a wide variety of stem cells within their niche during homeostatic maintenance and regeneration of distinct tissues. The focus is already moving away from defining cell-autonomous regulation of stem cell fate to documenting the dynamic interplay between cell types in the niche, extracellular matrix cues and the physical triggers that stimulate specific stem cell behaviours. As we have discussed, there have been several major developments in imaging systems that have made it possible to probe stem cell dynamics in tissues that were previously inaccessible. Although it is not currently possible to image all stem cell populations, further understanding of the molecular properties of specific stem cell populations will enable the generation of new transgenic lines to mark them. In addition, new imaging systems, such as optical projection tomography (Andrews et al., 2016) and even combining LSFM with two-photon microscopy (Truong et al., 2011), are currently being optimised for live imaging at higher resolution and faster capture rates. Thus, it might be possible in the near future to further increase imaging resolution and access to previously difficult to visualise stem cell niches.

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Competing interests
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