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2-photon microscopy for intra-cutaneous imaging of stem cell activity in mice

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Abstract

The adult skin is a typical example of a highly regenerative tissue. Terminally differentiated keratinocytes are shed from the external layers of the epidermis or extruded from the skin as part of the growing hair shaft on a daily basis. These are effectively replenished through the activity of skin-resident stem cells. Precise regulation of stem cell activity is critical for normal skin homeostasis or wound healing and irregular stem cell proliferation or differentiation can lead to skin disease. The scarcity and dynamic nature of stem cells presents a major challenge for elucidating their mechanism of action. To address this, we have recently established a system for visualizing stem cell activity, in real-time or long term, in the intact skin of live mice using 2-photon microscopy. The purpose of this review is to provide essential information to researchers who wish to incorporate 2-photon microscopy and live imaging into their experimental toolbox for studying aspects of skin and stem biology in the mouse model. We discuss fundamental principles of the method, instrumentation and basic experimental approaches to interrogate stem cell activity in the interfollicular epidermis and hair follicle.

Introduction

The replenishment of lost cells to maintain homeostasis and the repair of the skin after wounding rely on the activity of resident stem cells (1–3). Due to their unique properties of self-renewal and multipotency stem cells can be indispensable for normal skin function or extremely detrimental when they deviate from their standard activity (4–8). One of the major impediments on studying adult stem cells is that they usually constitute a relatively small fraction of the total population. While significant progress has been made in this front, with the identification of genes that are preferentially expressed in restricted populations that display stem cell properties in the skin, questions remain regarding the level of heterogeneity within these populations. Another significant challenge is that stem cell activity is by definition a highly dynamic process. While the microscopic analysis of frozen or paraffin embedded skin sections - processed by conventional histology and immunohistochemistry - has been the workhorse of dermatological research, this method is not always sufficient to

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Conflicts of Interests

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overcome the unique challenges of studying stem cell activity *in vivo*. Live imaging modalities such as 2-photon microscopy – especially when combined with powerful mouse genetic models - can provide critical insight into the mechanisms that govern stem cell activity, by enabling the visualization of stem cells within their native environment in the intact living skin (8). In contrast to other tissues, the skin being the most external organ in the adult body offers direct access for observation and requires minimal, non-invasive preparation for imaging (9). This review discusses recent methodological advances in the use of 2-photon microscopy for intra-cutaneous imaging of stem cell activity.

2-Photon microscopy: Principle and advantages

Two-photon laser scanning fluorescent microscopy (abbreviated here as 2-photon microscopy) was introduced in 1990 by Webb and colleagues to mark a turning point for the advancement of intravital imaging (10). 2-photon microscopy is an optical modality which relies on the use of a tunable, pulsed laser that emits light in the infrared spectrum (11). Similar to a confocal microscope, the laser raster-scans the sample and induces the excitation of fluorescent molecules. The major difference between confocal and 2-photon microscopy is defined by the mechanism of fluorophore excitation and the physical properties of infrared light (12). While a single photon in the visible excitation spectrum is sufficient to induce fluorescence, infrared light is less energetic; thus, to induce the transition of the fluorophore to its excited state two photons need to combine their energy by being absorbed at the same time. Such an event is naturally very rare but can be effectively achieved with the use of a high peak-power pulsed laser, which compresses light into intense packets, crowding the photons to increase the probability of two-photons being absorbed simultaneously by the fluorophore. This process provides a key advantage in that 2-photon excitation only occurs at the focal point defined by the objective lens. In contrast, in wide field and confocal microscopy single photons excite – albeit less efficiently – fluorescence continuously as they travel through the sample before reaching the focal plane; therefore, increasing background noise and inducing higher levels of photo-bleaching, photo-damage and photo-toxicity in the sample.

In practical terms, 2-photon microscopy offers four major advantages over other optical fluorescent modalities: 1) The single-plane excitation enables precise control of the contour of the visualized area and true optical sectioning, which together offer high three-dimensional resolution of the observed tissue. 2) Fluorescent proteins and probes have broad excitation spectra in the infrared space (13,14) 2-photon microscopy induces substantially less photo-damage, compared to confocal, which makes it feasible to visualize live tissues for extended periods of time with negligible impact to their normal physiology (15,16). 4) Infrared light scatters significantly less as it travels through the living matter, enabling imaging of cells and structures at much higher depths within the tissue (17). 5) The short-pulse lasers used in 2-photon microscopy are capable of generating contrast from internal tissue structures without the use of exogenous fluorescent probes. For example, in a process known as second harmonic generation (SHG), two photons interacting with non-centrosymmetric structures - such as collagen fibers and elastin, which are abundant in the skin - generate new photons with twice the energy and therefore half the wavelength (18,19). This can be collected as specific signal with the use of appropriate emission filters. These

properties make 2-photon microscopy an ideal optical method for dissecting the mechanisms of cellular activity in the skin, given its unique anatomy and stratified organization.

Intra-cutaneous imaging in the live mouse

2-photon microscopy has successfully been implemented in dermatological clinical research (20–25). Efforts to use the technology as a diagnostic tool by visualizing human skin are certainly exciting but mouse models are still invaluable for studying basic biological mechanisms of skin function and disease, especially when trying to understand processes as dynamic and complex as tissue regeneration. We have recently established a method for visualizing stem cell activity in the intact skin of living mice using 2-photon microscopy (8,26). A detailed description of our experimental protocol is available elsewhere (27); however, here we discuss important aspects of the method that will be useful to readers who wish to utilize a live imaging approach to address specific questions in basic skin and stem cell biology. These include instrumentation (Supplement), animal and tissue preparation and mouse genetic tools.

Animal and skin preparation for live imaging

In a typical live imaging experiment involving internal organs this step can be rather complicated and time consuming but this is not the case when imaging the skin (9). A number of different skin mounting apparatuses have been proposed to date and most of them share some common features and requirements (27–30). Anesthesia is one of those requirements, in order to keep the animal and the mounted skin stable for the duration of the experiment. Anesthesia can be easily achieved with injectable (ketamine/xylazine mix) or inhalable (isoflurane) anesthetic. To sustain and monitor the health of the mouse during the course of the experiment a temperature controlled heating plate and sensor are also required. In addition, a pump should also be considered to provide fluids and electrolytes intraperitoneally in a controlled manner, especially if the mouse will be imaged for extended periods of time.

Mice used for live imaging experiments should be bred into an albino background because pigment absorbs intensely in the infrared spectrum. This, may be detrimental to the health of the tissue following prolonged exposure but also produces strong autofluorescence which interferes with signal detection (28,31,32) The mouse skin requires very little preparation before mounting and we typically only remove the external hair shafts using a minimal amount of depilatory cream (Nair), which if carefully applied does not affect the normal activity of the keratinocytes and stem cells in the skin in any appreciable manner. Various skin areas including the ear, back, paw or tail can be easily mounted for imaging (Fig. 1). The ear skin can be particularly thin and tissue structures including the hair follicles are closer to the surface, which gives superior resolution compared to other areas of the skin. However, many hair follicles in the ear especially those closer to the tip do not display the same cycling characteristics as other sites. Conversely, the paw is devoid of hair follicles but is the only site where eccrine sweat glands can be found in the mouse skin. The back skin is routinely used for conventional histology and immunohistochemistry but the tissue is thicker compared to the ear and the hair follicles are difficult to be resolved in their entirety when in

full growth phase. The primary concern regardless the site of choice is to maintain stability throughout the experiment and keep the mounted area free of movements that can be caused by breathing or heartbeat - especially when imaging at sub-micron resolution - without compromising the normal physiology of the tissue.

For mounting, a depilated area of the skin is placed between a flat stable surface and a coverslip. We frequently use the ear because it offers a stable platform for mounting and manipulation. It should be noted that skin in the ear pinna is particularly thin and has hair follicles that display unique cycling characteristics; however, the area connecting the ear to the head more closely resembles the back skin and can be used instead. The use of a coverslip is preferred for two main reasons. First, the coverslip applies downward pressure to the mounted area of the skin and contributes to maintaining overall stability (however, too much pressure can inhibit normal blood flow, so caution should be exercised). Second, the coverslip flattens the surface of the skin, creating a single, starting focal plane from which optical sectioning can commence towards the deeper layers of the tissue. Most objective lenses have a correction collar to minimize spherical aberration caused by the different refractive index of the coverslip.

Visualization of stem cell activity in the interfollicular epidermis

The interfollicular epidermis is particularly attractive for live imaging studies, due to its stratified organization and thickness, which is less than 50 μm in the mouse skin and well within the range of what can be effectively resolved by 2-photon microscopy. To capture the activity of the basal epidermal progenitors that maintain the epidermis the first step is to choose the appropriate signal by which these cells can be visualized and distinguished among the general population. In recent years, a great number of transgenic mouse lines expressing exogenous fluorescent proteins that are suitable for skin research have been reported and many of them are available through commercial repositories. The keratinocytes in the interfollicular epidermis express different genes depending on their differentiation state. For example, keratins 5 and 14 are primarily expressed in the basal layer while involucrin and keratin 10 are expressed in committed keratinocytes undergoing differentiation (33–36). Fluorescent proteins expressed under the control of the respective gene promoters can serve as markers to visualize the individual cell types (37,38).

The use of constitutively expressed markers is not sufficient to study the activity and fate of individual stem cells in the epidermis. Instead a lineage tracing strategy can be implemented which typically involves the use of an inducible Cre-recombinase allele and a Cre reporter allele combined in the same mouse (39–42). Limited induction of the Cre-recombinase activity enables the stable expression of a fluorescent marker in few randomly distributed cells as well as their progeny. By monitoring labeled cells over time one can directly assess their self-renewal and differentiation capacity *in vivo*. Furthermore, the unique morphology and stratified tissue organization allows the unambiguous determination of the cell fate since the differentiation state of individual keratinocytes can be easily assessed based solely on their morphology and location within the different layers of the epidermis.

For these experiments, we have chosen a Cre reporter mouse strain which expresses a membrane-bound red fluorescent protein in all cells including the epidermal keratinocytes (43; Fig. 2A). Optical sectioning by 2-photon microscopy can easily resolve all the different layers of the epidermis. We combined this Cre reporter with an inducible Cre-recombinase allele that is expressed under the control of the keratin 14 promoter (44). Limited induction removes the floxed sequence of the red fluorescent tag along with the stop codon, which in turn induces the expression of a membrane-bound green fluorescent protein. Two days after induction the epidermis of the treated mouse is imaged and serial optical sections are acquired at 1–2 μm steps to capture the entire volume of the epidermis; starting from the surface until the bottom of the basal layer is reached. This is marked by the appearance of collagen-induced SHG signal. On this first time-point in the series single green-labeled basal keratinocytes that have undergone recombination can be visualized. By identifying and revisiting the exact same areas of the epidermis at regular intervals over the next few days, the same process can be repeated in order to directly evaluate the fate of the traced cells (26,27). At the end of the time-course the epidermal 3-D volumes can be reconstituted from the acquired image stacks of each time point and the activity of individual stem cells can be retrospectively evaluated.

Visualization of stem cell activity in the hair follicle

Hair follicles are self-contained mini-organs of the mammalian skin with a resident stem cell pool that is sufficient to periodically and stereotypically regenerate new hair appendages throughout life (45). In this process, which is known as the hair cycle and comprises of quiescent, growth and destruction phases (46); a small pool of hair follicle stem cells (47) generate a large number of progenitors which in turn differentiate into the seven lineages that make up the mature and growing hair shaft (38,48). A key question is how stem cells in the hair follicle contribute to the hair regeneration process. Using 2-photon microscopy we previously established the ability to visualize the process of hair regeneration in the intact skin of live mice, in real-time but also long term (8,26).

Dynamic stem cell activity taking place during hair regeneration can be visualized in the mouse, in real time by 2-photon time-lapse imaging. Since time-lapse imaging involves excitation of the sample at frequent intervals, a strong fluorescent reporter resistant to photo-bleaching is generally preferred. Furthermore a reporter that specifically localizes to the cell nucleus is also beneficial since it allows neighboring cells to be spatially resolved. Mice are chosen based on their respective age, since hair follicles are synchronized during the first 6–7 weeks of age and the time of each phase of the hair cycle can be predicted with relative high accuracy. Serial optical sections are typically acquired in 2–3 μm steps at a range that encompasses the entire volume of the hair follicles within the field of view; typically between 50–150 μm depending on the growth phase. The process is repeated at regular intervals for a desired total duration, which can extend to several hours depending on the imaging conditions, the state of anesthesia and the overall health of the mouse during the course of the experiment. Acquired data can be edited after the imaging session is concluded. Corresponding frames from each time point at the desired z-plane are arranged sequentially in a time series to form a movie, which can be qualitatively or quantitatively analyzed (Fig. 2B).

To elucidate the long-term contribution of hair follicle stem cells to hair growth a lineage tracing approach can be implemented and combined with frequent re-visits of the same hair follicles during the regeneration process (26,27). In such experiments and due to the unique physiology of the hair follicle, it's periodic regeneration process and the major morphogenetic changes that occur at different stages of the hair cycle, the timing of induction is most critical. For this we take advantage of the synchronized cycling of hair follicles in juvenile mice and treat with Tamoxifen at post-natal day 20, when the first hair cycle is concluded and the hair follicles are suspended for a brief time in their most minimal state during the first Telogen phase (Fig. 2C,D). A number of different promoters can be used at this stage to drive the expression of the Cre recombinase and label individual hair follicle stem cells, including Keratin 15, Keratin 19 and Lgr5 (48–50). The contribution of these stem cells can be evaluated by re-visiting the same follicles once they have entered the Anagen phase and by visualizing the localization of their progeny. Since a growing hair follicle is organized in concentric layers of basal undifferentiated and suprabasal differentiated lineages, each with a unique morphology, the differentiation state of each stem cell lineage and therefore their particular contribution to the regeneration process can be accurately assessed. Such initial studies showed that the Telogen hair follicle consists of a heterogeneous pool of progenitor cells, each with a unique activity and contribution during the hair cycle (26).

Considerations and future perspectives

2-photon microscopy is a powerful but relatively new tool in the arsenal of skin and stem cell biologists and as such there are certain considerations and limitations to be taken into account as well as tremendous opportunities for further development of the technique. Even though the method does not require any invasive procedures to visualize elements of the live mouse skin, the tissue as well as the animal are subject to preparatory treatments, such as chemical depilation, mechanical pressure and anesthesia, to name a few, which may have the potential to alter the normal physiology of the tissue. Appropriate controls should always be considered to minimize such concerns. Furthermore, as in most types of optical microscopy a source of signal, usually in the form of an exogenously expressed fluorescent protein, is required for visualization. This can limit the cell types within the skin that can be distinguished or introduce biases towards particular subpopulations, especially when inducible reporter systems are used. Even thought there is currently a limited list of transgenic mouse lines that can be used for live imaging of the skin the list is destined to expand rapidly, especially with the introduction of new genome editing tools such as CRISPR/Cas9 (51). Last, since the method is currently optimized for the murine system due to its genetic amenability questions will always persist regarding relevance to the human skin biology. Live imaging of intact human skin is indeed possible but relies exclusively in indiscriminate contrast mechanisms with low to no cell specificity. A possible adaptation of the technique would employ organotypic cultures established from human primary cells or organ-cultured explants from adult human skin that can easily be genetically manipulated to introduce a variety of fluorescent reporters (52–55). It is certain that broader adaptation of 2-photon microscopy and live imaging approaches will greatly augment dermatological research in years to come.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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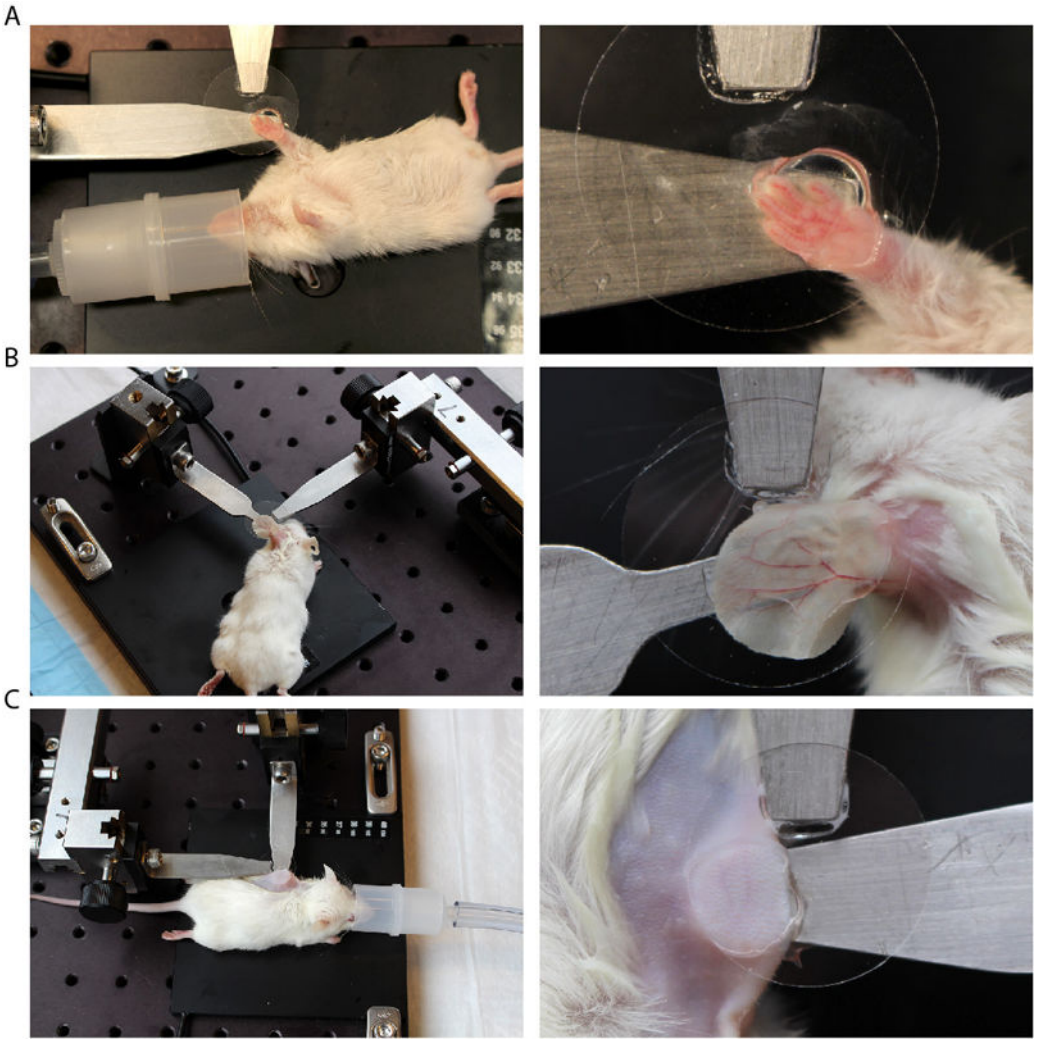

Figure 1.
Example of a mounting apparatus for immobilizing (A) the paw, (B) ear and (C) back skin of an anesthetized mouse for intracutaneous imaging.

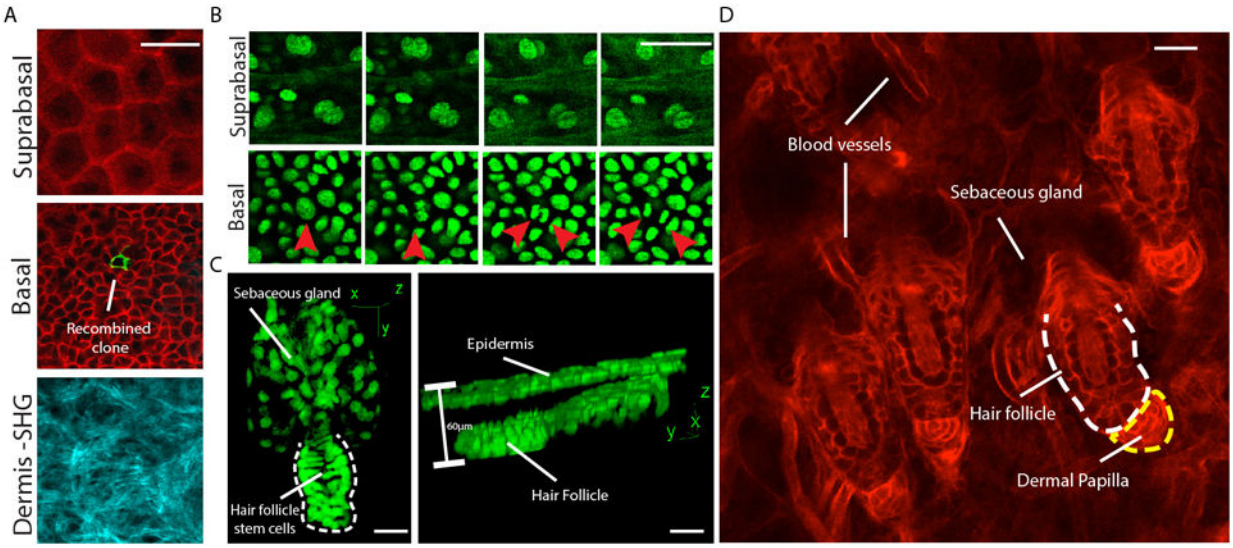

Figure 2.

(A) Single optical planes depicting different layers of the interfollicular epidermis. A Cre reporter mouse strain was used which ubiquitously expresses a membrane-bound RFP that converts to membrane-bound GFP (mTmG) after recombination from an inducible Keratin 14-driven Cre recombinase. (B) Time-sequence of keratinocyte activity in the corresponding basal and suprabasal layers of the interfollicular epidermis. A mouse strain was used that expresses GFP fused to histone H2B under the control of the Keratin 14 promoter. Timepoints are 20 min apart and red arrowheads point to a stem cell division in the basal layer. (C) 3D renderings of Telogen hair follicles. (D) A single optical plane from within the dermis showing a group of hair follicles and other cellular elements of the skin. Scale bars: 20 μm.