

Invited Mini Review

Adult stem cell lineage tracing and deep tissue imaging

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Lineage tracing is a widely used method for understanding cellular dynamics in multicellular organisms during processes such as development, adult tissue maintenance, injury repair and tumorigenesis. Advances in tracing or tracking methods, from light microscopy-based live cell tracking to fluorescent label-tracing with two-photon microscopy, together with emerging tissue clearing strategies and intravital imaging approaches have enabled scientists to decipher adult stem and progenitor cell properties in various tissues and in a wide variety of biological processes. Although technical advances have enabled time-controlled genetic labeling and simultaneous live imaging, a number of obstacles still need to be overcome. In this review, we aim to provide an in-depth description of the traditional use of lineage tracing as well as current strategies and upcoming new methods of labeling and imaging. [BMB Reports 2015; 48(12): 655-667]

FROM EARLY CELL TRACKING TO TODAY'S GENETIC LINEAGE TRACING

How an organism develops, starting from fertilization, is a fundamental question in biology. In the 19th century, conventional embryology described the development of the embryo from fertilization through the various stages of development. Although the fascinating nature of embryonic development was revealed, its description remained at the level of gross morphological changes due to the lack of modern devices as well as the complexity of developmental processes involving millions or billions of cells. In 1974, Sidney Brenner first started investigating the simple invertebrate organism *Caenorhabditis elegans* (*C. elegans*) in order to describe development at cellular resolution (1) (Fig. 1A). Its easily visible embryonic development allowed non-invasive microscopic analysis of developmental processes that remained inaccessible to scientists in

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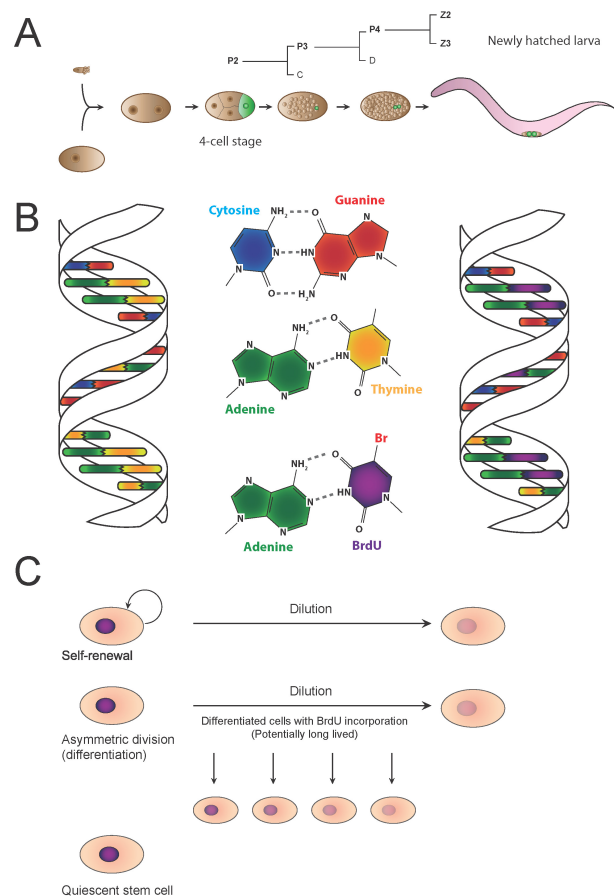


Fig. 1. *C. elegans* early development and BrdU tracing. (A) Schematic representation of *C. elegans* embryonic development. The germline develops from one single primordial germ cell (PGC) which appears in the early embryo at the 4-cell stage. This PGC will divide and give rise to the two PGCs Z2 and Z3. These PGCs will not divide further until after hatching. Postembryonic divisions result in variable, non-reproducible placement of the daughter cells (3, 4). (B) Illustration of bromodeoxyuridine (BrdU), a synthetic nucleoside that is analogous to thymidine, and how it is incorporated into the genome. (C) Representation of how BrdU is diluted during tracing. Rapid self-renewal of the stem cells (top row) will lead to dilution of BrdU. In contrast, quiescent stem cells (bottom row) will retain the BrdU label. During asymmetric self-renewal the progenitor cells divide and give rise to differentiated cells, leading to a dilution of BrdU.

other organisms. This enabled extensive lineage tracking studies and revealed that at early developmental stages the fate of a specific cell is invariably conserved between individuals. Furthermore, each cell undergoes a defined number of divisions thus resulting in a defined number of somatic cells in the adult (hermaphrodite: 959; male: 1031) (2-4). Nevertheless, limitations of the imaging technology at that time hindered scientists from tracking cell divisions in the later embryo. Thus, the advancement of imaging technology to visualize cellular events was predicted to lead to a deeper understanding of developmental processes.

The labeling of specific cells is one method employed to visualize subsequent cellular events. This represents an improved tracking strategy for the later stages of development, when millions of cells are present. A simple example is the labeling of proliferating cells by incorporation of radioactive nucleoside or nucleoside analogues such as 5-bromo-2'deoxyuridine (BrdU) (Fig. 1B). BrdU had first been described as an antagonist of the terminal steps of DNA-thymine synthesis in 1958 by Kit *et al.* (5). The use of BrdU incorporation for cell lineage tracing experiments became possible following the development of monoclonal antibodies against BrdU-containing DNA by Gratzner in 1982 (6). In the following years, BrdU incorporation was used extensively to study cell proliferation (7-12) and to label proliferative cells in order to trace their fates in various organ systems (13-17). However, BrdU is diluted following each cell division and is eventually lost in rapidly dividing cells as newly synthesized, unlabeled nucleotides are incorporated into the genome (Fig. 1C). Therefore, BrdU incorporation is useful only in studying cell proliferation, short term lineage hierarchy and label-retention.

Recent advances in genetic manipulation techniques have facilitated the development of genetic labeling strategies that allow the long term labeling of both cycling and quiescent stem cells as well as the permanent tracing of all their descendants. One of the most important tools in performing lineage tracing studies in mice is the Cre/loxP site-specific recombination system first described in the bacteriophage P1 in 1984 by Abremski *et al.* (18). This system allows genetic manipulation by Cre-mediated recombination of LoxP sites integrated into the genomic regions of interest. Further development of a ligand-dependent Cre recombinase – CreERT2 – additionally enabled scientists to temporally control the recombinase activity through the administration of inducing agents (19). For lineage tracing studies, two genetic tools needed to be combined: i) CreERT2 expression under the control of a cell type-specific promoter and ii) reporter gene expression that can be induced upon Cre activity, thus allowing heritable expression of active reporter genes in all progeny. Initially, β -galactosidase (LacZ) was used as a reporter gene (20, 21) (Fig. 2A). Nowadays, single (22-24) to multi-color fluorescent proteins (25-27) are widely utilized for lineage tracing (Fig. 2B, C).

In this review we will discuss recent lineage tracing studies and quantification tools as well as novel live imaging ap-

proaches to give an overview of existing techniques and to outline future directions in the field of modern adult stem cell tracking and fate choice studies.

MODERN LINEAGE TRACING STUDIES IN ADULT TISSUES

Recent lineage tracing studies with temporally regulated CreER recombinase and reporter lines have revealed details about the

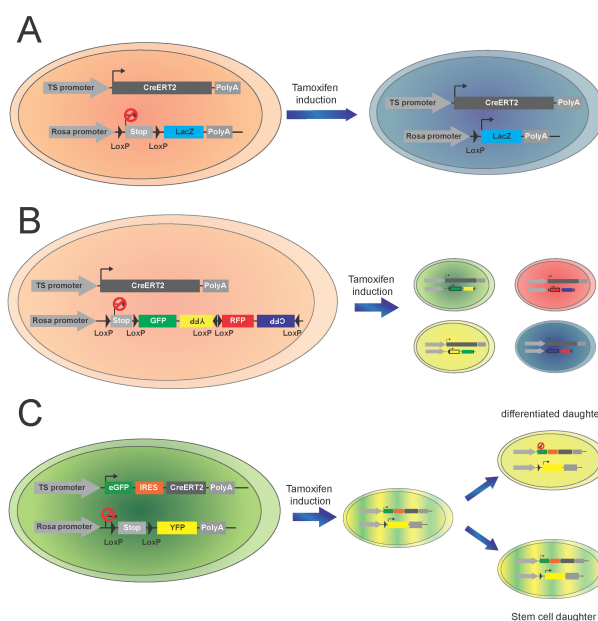


Fig. 2. Examples of different reporter lines. (A) LacZ reporter system in which Cre-mediated excision of the STOP cassette results in lacZ expression, which can later be visualized by X-gal staining. The expression of Cre recombinase is driven by a cell-type or tissue specific (TS) promoter. Tamoxifen administration induces recombination and subsequent LacZ expression in CreERT2 expressing cells. The genetic label is inherited by all daughter cells allowing tracing of the progeny of individual labelled cells. (B) Multi-color reporter. In this system the administration of tamoxifen leads to random recombination of four fluorescent proteins (GFP, YFP, CFP and RFP). Subsequently, the cells expressing CreERT2 under a tissue specific (TS) promoter will also randomly express one of the four colors. The genetic label is inherited by all daughter cells allowing tracing of the progeny of individual labelled cells in one of four colors. (C) eGFP-IRES-CreERT2 cassette. Expression of GFP by a cell-type or tissue specific (TS) promoter mediates green fluorescent labeling of the cell type of interest. The bicistronic eGFP-IRES-CreERT2 construct allows for inducible recombination in the GFP labelled cell population. Combined with a fluorescent reporter, this results in the expression of a second fluorescent protein under a ubiquitous promoter (here Rosa-YFP). In a stem cell that goes through asymmetric division, the daughter cell that remains a stem cell will express both colors (from the ubiquitous and cell-type specific promoter) while the daughter cell that differentiates and thereby turns off the cell-type specific promoter will only be labeled in one color (driven by the ubiquitous promoter).

homeostatic turnover of multiple tissues and about underlying cellular plasticity in homeostasis, injury response and cancer formation. An excellent example is the study of *Lgr5*-expressing stem cells in the small intestine and colon (28). In a previous study, the group of Hans Clevers identified *Lgr5* as a Wnt target gene whose expression is spatially restricted to the base of intestinal crypts (29, 30). They then designed a bicistronic construct – GFP-IRES-CreERT2 – to be inserted into the endogenous *Lgr5* locus, which allowed expression of 2 useful proteins (GFP for visualization and CreERT2 for time-controlled recombination) under the control of the *Lgr5* promoter (See Fig. 2C for an example of this bicistronic construct in combination with *Rosa-YFP* reporter). Using this novel strategy combined with the *Rosa26-LacZ* reporter they showed that the *Lgr5*⁺ crypt base columnar (CBC) cell population maintains itself for over a year and gives rise to all lineages present in the intestinal epithelium, therefore identifying the *Lgr5*⁺ population as intestinal stem cells (28) (Fig. 3). Similar lineage tracing analysis identified additional *Lgr5*⁺ stem cell populations in other tissues such as the colon, the hair follicle and the pylorus of the stomach (16, 31). These studies nicely illustrate

the utility of lineage tracing as a tool to elucidate the stem cell properties of potential stem cell populations.

IMAGING TECHNOLOGIES ENHANCE TISSUE-WIDE LINEAGE TRACING STUDIES

Unlike the highly ordered epithelium of the intestine, other epithelial structures (e.g. in lung, pancreas, liver and mammary glands) have a highly branched morphology that necessitates the use of sophisticated 3D imaging of thick tissue to analyze the entire lineage tracing event. In today's confocal microscopes, a selective filter only allows light of the desired wavelength to pass while a pinhole before the detector blocks any out-of-focus light to generate the corresponding emission image of the excited specimen spot. The resulting image is generated point by point, allowing in-focus imaging from selected specimen depths. Subsequent 3D-reconstruction can help in turn to identify stem- or progenitor cell-derived clonal expansions in lineage tracing experiments. However, the limitations of confocal laser scanning microscopy include photobleaching, laser light scattering, emission light scattering and imaging time (32-34).

The development of 2-photon microscopy aimed to circumvent these photo bleaching and laser light scattering problems by using photons of longer wavelengths (lower energy) that can excite fluorescent proteins or dyes only in a defined focal plane (34). Where the paths of both photon beams cross, simultaneous absorption of the individual photons leads to a selective increase in photon energy (35-37) which subsequently reaches the required energy to excite the fluorescent dye or protein of interest.

Light-sheet microscopy represents another alternative to conventional confocal microscopy, with improved imaging speed due to illumination and detection of entire focal planes at the same time (Table 1). This technology presents unprecedented low photo toxicity at very high image acquisition speed optimal for live imaging. The development of smaller light sheets, for higher axial excitation resolution, in combination with higher numerical aperture (NA) lenses for higher axial detection resolution enabled the fast detection of molecular processes like transcription factor binding kinetics in up to 35 μm thick live mouse embryonic stem cell spheroids (38). Alternatively, using lower NA lenses and thicker light sheets, this technology can be used to image entire small organisms or organs while maintaining high speed image acquisition, though at lower resolution (For technical details see: 38-42). Due to its superior performance in cell viability, light sheet microscopy has been used extensively for live imaging of developmental processes in e.g. *C. elegans* and *Drosophila melanogaster*, *Tribolium castaneum*, zebrafish or mouse embryos (38, 40, 41, 43-49). In the field of adult stem cell lineage tracing, the light sheet microscopy technology holds particular promises in live tracking of stem cell mediated homeostasis and for the observation of injury mediated repair mechanisms.

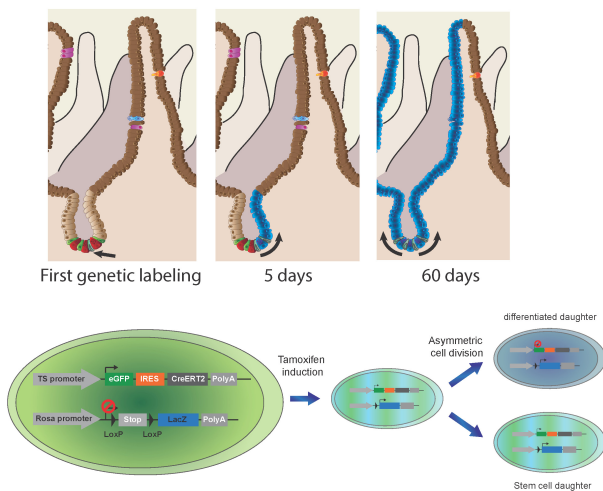


Fig. 3. Lineage tracing of *Lgr5*⁺ intestinal stem cells using the lacZ reporter system. Lineage tracing of *Lgr5*⁺ stem cells. An eGFP-coupled, tamoxifen inducible knock-in mouse is used in which the expression of eGFP and CreERT2 is driven by the LGR5 promoter. All *Lgr5*⁺ stem cells express eGFP. Following the administration of tamoxifen, recombined stem cells express *Lgr5* promoter driven eGFP and ubiquitous promoter driven LacZ. Upon division, daughter cells of *Lgr5*⁺ stem cells either remain as stem cells expressing both GFP and lacZ, or differentiate turning off the expression of *Lgr5* expressing only the lacZ reporter gene. LacZ⁺ cells derived from labelled *Lgr5*⁺ stem cells quickly move upwards towards the villi (day 5). Neutral drift within the stem cell zone results in complete labeling of the entire crypt within several weeks. All cells of the adjacent villi have been replaced by labelled progeny of one initially labelled *Lgr5*⁺ stem cell (day 60).

Table 1. Fluorescence Microscopy for Lineage Tracing Imaging

	Standard Fluorescence Microscope	Laser scanning Confocal Microscope	2-photon Confocal Microscope	Light Sheet Microscope
Imaging depth	All (Out of focus because of lacking focal plane selection)	Up to 500 μm	Up to 1 mm	Up to 500 μm
Image plane selection	No	Yes	Yes	Yes
Photo bleaching	Medium – High (LED light for reduced bleaching)	Medium	Low	Very Low (Due to increased imaging speed and selected illumination)
Cell Viability	Low – High (LED light for increased cell viability)	High	High	Very High (Due to increased imaging speed and selected illumination)
Excitation	All	Entire specimen in objective focal axis	Limited to objective focal volume	Entire specimen in focal plane
Laser light scattering	N/A	High	Low	Very high
Emission light scattering	N/A	Dependent on specimen and image depth	Dependent on specimen and image depth	Dependent on specimen, mounting, and image depth
Price	Low - High	High	Very High (Laser)	Low - Very High
Main Limitations	Imaging Depth, Z-resolution	Imaging time, photo-damage	Imaging time, heat, price	Imaging depth (Steric hindrance), price, specimen mounting is crucial, implementation of most recent optimizations ongoing
Main advantages	Accessibility, very good for 2D of thin sections, additional deconvolution	Accessibility, 3D imaging	Imaging depth, low photodamage	Imaging speed, very low photodamage, specimen size

A few stem cell lineage tracing studies have utilized this novel technology. One example is the identification of perivascular collagen1 α 1 cells as the main source of the cellular composition of the fibrotic scar after contusive spinal cord injury (50). Commercially available light sheet microscopes and readily available access to this novel technology in imaging facilities will speed up adult stem cell lineage tracing experiments.

Currently light sheet microscopy is being used for small organs or organisms (up to a few hundred μm) at high axial resolution using high NA lenses. However, the use of these lenses results in steric hindrance due to the required working distance of each lens (38). Therefore, Imaging of larger specimens (up to cm^2) is performed using low NA lenses at a lower axial resolution. The main obstacle of light sheet microscopy as well as all other imaging techniques aiming at deep tissue visualization is optical heterogeneity of the specimen and the resulting light refraction, which causes light scattering and reduces the number of photons reaching the detector or camera.

To circumvent this problem, another strategy that aims to aid visualization of thick specimens by reducing the light scattering properties of intact tissues and therefore increasing overall optical transparency has been developed. In 1914, Werner Spalteholz performed pioneering studies in this field by using organic solvents to reduce light scattering within tissues (51).

Commercially available mounting reagents, e.g. RapiClear[®] (RC), can improve the light permeability of samples by minimizing light scattering at the interface between coverslip and specimen and within the specimen itself. The refractive index (RI) of RC 1.52 is around 1.52nD, close to that of lipid membranes, which are a major source of light scattering in the tissue. Additionally, the RI of RC 1.52 is close to that of glass. If oil lenses are used, all RIs on the path from the samples to the cover slip and objective are consistent, increasing the resolving power as well as signal brightness.

Several additional clearing reagents have been described in recent years to perform clearing and subsequent 3D imaging of whole organs (39, 52, 53). These techniques have further been optimized to reduce fluorescent quenching during the process of clearing (54-57). All of these tissue clearing strategies aimed at increasing the light permeability of tissues in order to visualize expressed fluorescent proteins, but limited antibody penetration poses another challenge to the molecular interrogation of intact tissues that needed to be overcome. Chung et al developed an ionic extraction technique, named CLARITY (originally an acronym for Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/*In situ* hybridization-compatible Tissue-hYdrogel), to remove the lipid bilayer of cells while maintaining the structural integrity of the

Table 2. Tissue clearing summary

	BABB (Becker et al, 2008; Dent et al, 1989; Doodt et al, 2007; Spalteholz, 1914)	Scale (Hama et al, 2011)	Tetrahydrofuran (THF) (Ertürk et al, 2012b)	3DISCO (Becker et al, 2012; Ertürk et al, 2012a)	CLARITY (Chung et al, 2013; Lee et al, 2014; Tomer et al, 2014)	PACT (passive CLARITY technique) / PARS (perfusion-assisted agent release in situ) (Yang et al, 2014)
Fixation method and other tissue preparation	PFA perfusion	PFA perfusion	PFA perfusion	PFA perfusion	PFA, acrylamide, bis-acrylamide, VA044 perfusion	PFA perfusion and subsequent hydrogel/ initiator perfusion
Dehydration	Yes	No	Yes	Yes	No	No
Clearing solution	Benzylalcohol, benzylbenzoate	ScaleA2: Urea, glycerol, Triton X-100	THF, dichloromethane (DCM), benzylalcohol, benzylbenzoate	THF, DCM, dibenzyl ether (DBE)	Active transport organ-electrophoresis approach -electrophoretic tissue clearing (ETC) in sodium borate buffer containing 4% SDS	Up to 2 weeks 8%SDS in PBS, pH 7.5 at 37-42°C followed by extensive PBS perfusion washing over 2-3 days
Time of protocol	Up to 3 days or more	3 days - several weeks (- months)	~ 1 day	~ 1 day	~ 7-21 days	2-3 days for most organs, 1-2 weeks for whole brain
Tissue clearing principle (RI matching)	Dehydration	Increased RI of aqueous phase	Dehydration and lipid removal (THF)	Dehydration and lipid removal (DCM)	Ionic lipid extraction (Passive or electrophoretic)	Ionic lipid extraction
Fluorescence quenching	High (Dehydration)	Minimal	High (Dehydration)	Continuous quenching in final clearing solution	No	No
Main Disadvantages	Not compatible with tissues containing a high degree of lipids, fluorescence quenching, autofluorescence, benzylbenzoate and benzylalcohol are toxic and dissolve plastic	Long clearing process, fragility of cleared samples, sample expansion	Impaired ultrastructure, loss of cellular and molecular information due to lipid removal, benzylbenzoate and benzylalcohol are toxic and dissolve plastic	Quenching in final clearing solution requires immediate imaging, loss of cellular and molecular information due to lipid removal	Passive: Long clearing Electrophoretic: Additional equipment needed (platinum electrodes), possible epitope loss, heating	Relatively slow for large organs like whole brain (compared to BABB, Scale, THF or 3DISCO), perfusion chamber required
Main Advantages	Accessibility to reagents	Signal preservation of fluorescent proteins, adjustable formula for various organs, low toxicity of chemicals	Reduced fluorescence quenching, antibody use possible, but long incubation times required	Relatively low fluorescence quenching, lipid removal, antibody use possible, but long incubation times required	Preservation of biological information by providing physical framework, no quenching due to ionic extraction technique, deep antibody penetration with long incubation times	Superior to CLARITY due to lack of electrophoresis and reduction of tissue degradation, more cost effective than CLARITY, reduced tissue swelling, fast simultaneous clearing of multiple tissues

tissue (58). First, the tissue of interest gets perfused with a combination of hydrogel monomers, formaldehyde and polymerization initiators (at 4°C). After incubation at 37°C, the hydrogel monomers polymerize, incorporating biomolecules within the mesh of hydrogel and stabilizing the 3D structure of the tissue. In the second step, lipids and other unbound biomolecules can be extracted by active electrophoresis. Besides the obvious effect of optical tissue clearance, the hydrogel mesh in combination with lipid extraction allows increased antibody penetration and reduced loss of proteins compared to other clearing or permeabilization protocols (42, 59). Yang et al reported a perfusion-based modification of the CLARITY protocol with superior tissue clearing speed and reduced risk of tissue degradation or overheating (PACT: Passive CLARITY Technique / PARS: Perfusion-assisted Agent Release *in Situ*) (60) (Table 2). The application of this technique in combination with modern deep tissue imaging and long term lineage tracing studies of potential organ-specific progenitor or adult stem cell populations will enable 3D analysis in various tissues.

MATHEMATICAL MODELING TO UNDERSTAND UNDERLYING CELLULAR DYNAMICS

The development of better 3D imaging techniques resulted in the capability to acquire induced clonal expansion data from lineage tracing that enables additional retrospective analysis of clonal behavior. Although analysis of individual time points after labeling of specific stem cell populations only reveals restricted information about the underlying clonal expansion, the consecutive analysis of multiple time points and subsequent integration of the acquired data allows the development of mathematical models which permit a glimpse of clonal behavior on a population level. In other words, the acquisition of clonal expansion data at multiple time points and in multiple biological replicates provides the dynamic information required to understand both the kinetics of cell proliferation and stem cell fate behavior. Two conceptual approaches are frequently taken to infer information about these two biological concepts. Here we outline the main strategies that can be followed to infer information about biological processes by utilizing mathematical modeling approaches in combination with adult stem cell lineage tracing experiments.

Analysis on a population level

To decipher the kinetics of cell proliferation by lineage tracing analysis, scientists and mathematicians use quantitative population average measurements following a continuous labeling approach such as BrdU incorporation. Following the BrdU incorporation, over time all newly generated cells are going to be labeled, ultimately resulting in labeling of all cells at a rate that depends on both the rate of stem cell divisions (λ) and the rate of differentiated cell loss (Ω). Assuming a simplified two compartment model in which cell divisions and cell loss occur independently of each other, the behavior

of the labeled cell population is described by the fraction of labeled cells over all cells as a function of chase time: $f(t) = (1 - pe^{-2\lambda t} - (1-p)e^{-\Omega t}) \times 100\%$, with the fraction of proliferative cells $p = 1/(1 + \lambda/\Omega)$ (61). For homeostatic conditions, the curve resulting from this model is entirely described by only cell division (λ) and loss rate (Ω), and average information about both biological processes can be inferred using mathematical modeling in combination with this simple labeling approach and quantitative data acquisition. Similarly, label dilution assays can be utilized to describe proliferation kinetics. However, as both strategies rely on population average measurements, these approaches can only give information about the stem cell behavior on a population basis. Beyond the average information on cell division and extinction, individual cell fate decisions underlying the observable population dynamics should be interrogated using a clonal approach.

Analysis on a clonal level

Understanding individual stem cell fate behavior requires clonal assays which in many cases are achieved by genetic labeling, utilizing the inducible CreERT2 system. Assuming that clonal labeling is achieved, a snapshot of clone size and cellular composition of multiple clones at a given time point should reflect all possible fate paths that led to the recorded clonal data. By increasing the number of analyzed clones and time points, the empirically observed frequency of clones in a certain fate path becomes proportional to the actual chance of that particular fate path. The application of mathematical models in this context is dependent on the initial hypothesis that needs to be tested to dissect a given biological process. One very good example of how mathematical modeling can be applied to clonal data is the description of neutral drift within the $Lgr5^+$ intestinal stem cell population towards monoclonality. The mathematical principles that underlie this analysis have been described in detail in the original paper by Snippert et al and in a subsequent paper by the group of Jacco van Rheenen (26, 62). Here we want to summarize the most important criteria that need to be considered for the experimental design. For this type of study it is important that the induction frequency is low enough so that subsequent tracing events have a defined high chance of being the progeny of a single labeled cell. Additionally, the scoring method needs to be well defined to address the hypothesis in question. Scoring all clones by size can be helpful to decipher the variability in fate paths that a single cell can follow, but to understand stem cell dynamics in detail it might be important to score clones by taking additional criteria into consideration. In this example, to decipher stem cell population behavior on a clonal level, all clones that have entirely left the stem cell niche (i.e. no more $Lgr5^+$ stem cell within the clone) and therefore ceased to contribute to the stem cell population have been considered as depleted clones. Clones that consist of 6 $Lgr5^+$ and 10 $Lgr5^{dim}$ cells were scored as a clone size of 6. This simplified approach allows for more

detailed analysis of the stem cell compartment by discarding potentially misleading clone information caused by the high proliferative turnover of the transit amplifying cell population. The resulting clone size distribution describes the clonal behavior within the stem cell population.

Other examples of how lineage tracing experiments in combination with quantitative analysis have contributed to our current knowledge of adult stem cell behavior can be found in multiple studies. In 2007, the group of Philip H. Jones in collaboration with Benjamin D. Simons showed that in mouse tail epidermis only one type of progenitor cell maintains homeostasis, in contrast to the proposed model of two distinct populations of self-renewing stem cells and transit amplifying progenitor cells (63). In the following years, Benjamin Simons has worked together with multiple groups aiming to unravel the underlying cellular mechanisms that govern homeostasis, injury response and malignancies in multiple tissues. His collaborative studies have shed light on adult stem cell behavior in the ear epidermis, the esophagus (64, 65) and the intestinal epithelia (26, 66, 67) in mice. Other studies included the analysis of stem cell behavior in the mouse germ line (68), prostate (69), skin and intestinal tumors (70-72). These examples demonstrate that quantitative analysis of lineage tracing events expands the biological significance of the acquired data, so providing new insights into adult stem cell differentiation and self-renewal.

LIVE CELL IMAGING *IN VITRO* AND *IN VIVO*

In contrast to the retrospective analysis of clonal expansion based on lineage tracing, live imaging allows continuous direct observation of the cells or organism of interest, for a certain period of time. Up until the late 1980s, the conditions during the live imaging process were incompatible with the maintenance of mammalian cells for more than a few hours, thus severely limiting the information that could be gained. In order to study the hierarchy of neural progenitors, Sally Temple placed her microscope inside the incubator and for the first time mammalian cells could be imaged for a longer time period. This was the beginning of the development of incubation chambers (73, 74). Today, computerized incubation chambers, microscope stages and major technical advances in the development of microscopes (e.g. two-photon and spinning disc microscopes) combined with rapidly expressed, bright fluorescent proteins have allowed the use of live imaging to monitor biological events in real time (73, 75-78). On a cellular level, the application of live cell imaging has been used to monitor the phenotype of rat retinal progenitor cells prior to mitosis in order to determine whether characteristic phenotypes could predict the fate choice (79). At a larger scale, *in toto* (Latin: as a whole) imaging is commonly applied. As the name implies, the aim is to image and track every single cell movement and division that forms a tissue or organ (80). Since its development in 2003 by Sean Megason and

Scott Fraser, it has been extensively used to study embryonic development. Model organisms such as zebrafish and *C. elegans* are commonly used due to their suitability for imaging and genetic manipulation, as both zebrafish embryos and *C. elegans* are small and transparent (81).

While zebrafish embryos develop freely outside of their mother in water, mouse embryonic development takes place inside the uterus. The developmental stages prior to implantation have been extensively studied, as mouse pre-implantation embryos can be isolated and their development can be observed *in vitro* by live imaging (82-84). Conversely, post implantation events have largely remained elusive as implanted embryos are inaccessible for direct observation. Recently the group of Zernicka-Goetz developed a novel *in vitro* culture system that allows the live imaging of the previously inaccessible implantation process (85). This exemplifies how novel *in vitro* culture systems in combination with live imaging can provide knowledge of biological processes and events that would otherwise be inaccessible *in vivo*.

Another example of how *In vitro* studies can provide new insights into adult stem cell behavior is the recently developed long term culture of organoids. The organoid culture system, maintained by actively cycling adult stem cells, provides a novel platform to study biological processes involved in the regulation of adult tissue specific stem cells. Organoids are self-organizing 3D structures whose architecture and physiological properties closely resemble the *in vivo* tissue (86). The development of genetic tool kits, such as retroviral transduction (87), bacterial artificial chromosome (BAC)-transgenesis (88) and CRISPR/Cas genome engineering (89) has allowed cell type- or gene-specific labeling with fluorescent proteins and functional genetics to be performed in this *in vitro* culture system to study stem cell maintenance, cell fate decisions and cell-cell interactions in combination with live imaging (86, 90).

INTRAVITAL IMAGING (*IN VIVO* LIVE CELL IMAGING)

The development of advanced *in vitro* culture systems has enabled studies of *in vivo* processes otherwise hidden from sight. An alternative has been the development of tools permitting imaging to be performed *in vivo* to study biological events in living animals. The ability of the firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) to biochemically generate light using luciferase enzymes inspired scientists to develop a reporter system, using the same enzymes, allowing the non-invasive tracking of cells (91, 92). To date, several transgenic luciferase reporter mice have been generated and used to study metastasis, gene expression and bacterial and viral infections (92, 93). Although *in vivo* bioluminescence imaging has minimal background and a high signal-to-noise ratio, it lacks cellular resolution (92, 94). This issue can be circumvented by using confocal and two-photon microscopy in combination with fluorescent reporter mice. The innovation of imaging windows (95) allowed Lehr et al to be among the first to perform intra-

vital imaging over multiple days (96, 97). Ever since, imaging windows for skin, cranial, mammary and abdominal imaging have been developed (98-101) and intravital imaging has been applied to investigate various biological processes in several different research areas, for example angiogenesis, neurobiology, immunology, and cancer biology (99, 102-109). For long-term imaging (days to weeks), tattooed reference marks in combination with vascular and extracellular matrix structures can be used as landmarks to re-identify previously imaged cells (110-112).

An excellent example of the use of intravital imaging in adult stem cell research is from the group of Jacco van Rheenen. Previous studies utilizing lineage tracing managed to delineate the hierarchical organization of squamous skin tumors, intestinal adenomas and glioblastomas (72, 113, 114). Nonetheless, the static images fail to address the issue of plasticity and individual clone behavior. Using the *Lgr5-CreERT2;confetti* mouse line (26), van Rheenen and his team confirmed the previously observed neutral drift model and in addition provided insight into the short-term dynamics of the *Lgr5*⁺ stem cells. Even though all *Lgr5*⁺ cells possess long-term self-renewal potential, the stem cells located at the crypt base, called 'central stem cells', have a survival advantage over the 'border stem cells', which are located in the upper part of the niche and can be displaced into the transit-amplifying domain following the division of adjacent cells (107) (Fig. 4).

OUTLOOK

To date, the technical advancement of microscopes (in both hardware and software) as well as the development of sophisticated molecular and genetic tools has led to a parallel development of live imaging and lineage tracing. In recent years the two have started to come together, a synergy that has led to novel insights ranging from developmental processes to cellular hierarchies within adult tissues and tumors. An obstacle still remaining is the long-term, intravital imaging of deep-tis-

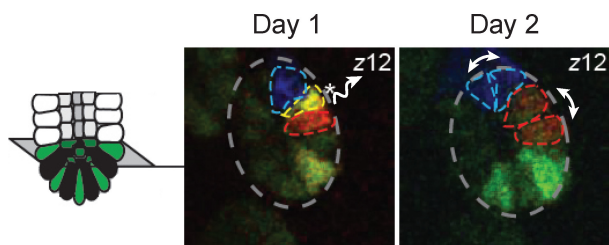


Fig. 4. Intravital imaging of the intestinal stem cell niche. Intravital images of the same intestinal crypt after tamoxifen mediated labeling of *Lgr5-CreERT2* expressing stem cells on a confetti reporter background. The panels illustrate how two clones (red and blue) expand on dispense of a yellow clone, which gets pushed out of the stem cell zone.

sue, the solution of which would allow the observation of adult stem cell dynamics directly inside organs and tissues. Until this issue is resolved, the development of advanced *in vitro* culture systems which fully or partially recapitulate the *in vivo* situation, together with the innovation of new imaging windows, will be of paramount importance. Choosing the appropriate method best suited for the purpose of the experiment is crucial to achieve reproducible results and to draw solid conclusions from the observations.

Despite the existing technical obstacles, there are prospective new techniques and systems providing other highly interesting options. One is magnetic resonance imaging (MRI). It is the least invasive imaging technique (115) and can be applied to large volumes or whole organisms. In addition, it is safe enough to be used as a diagnostic tool (78). However, advanced experimental machines allowing near single-cell resolution in a spatiotemporal manner remain very expensive and are therefore not widely available (116, 117). Another major limitation is low contrast and signal strength. To overcome this issue, scientists have been using contrasting agents usually based on gadolinium (Gd^{3+}) (118). To use this technique for the purpose of adult stem cell lineage tracing, new genetic labels for MRI-contrasting agents can be used instead of fluorescent (e.g. YFP) or colorimetric (e.g. LacZ) labels. One approach is to utilize divalent metal ion transporters (DMT1) which facilitate the transport of Mn^{2+} and metabolically biotinylated cell surface protein, which can then be detected using a streptavidin-horseradish peroxidase conjugate in conjunction with a peroxidase-sensitive gadolinium agent (119). Recently, an *Oatp1a1* reporter has been described by Patrick *et al.* This transport protein mediates the uptake of Gd^{3+} (in the form of gadolinium-ethoxybenzyl diethylenetriamine pentaacetic acid), a clinically approved contrast agent for MRI (120). Genetic modification to generate ferritin-expressing cells has been shown to increase the contrast (121-123). Superparamagnetic iron oxide nanoparticles (SPIONs) have been used as contrast agents to label and track stem cells in rabbit (124), rat (125, 126) and human patients (127, 128).

Another technical advancement comes from the group of F. Levent Degertekin, which has developed a camera 1.5 mm in size. Still to be tested on animals, it holds the potential for taking 3D images from inside veins and arteries (129). This type of miniaturization of cameras and detectors, with the option of adding guiding wires or possibly implantation, even if transient, could aid and expand the field of vision for live imaging as well as lineage tracing. Recently, the group of Hongjie Dai developed a method utilizing nanotubes with intrinsic photoluminescence to image the mouse cerebral vasculature without any invasive procedures (except the injection of the nanotubes) (130). These two examples demonstrate how the development of micro- and nanotechnology can facilitate and enable previously unknown imaging possibilities.

Transparency is one of the strongest advantages of using zebrafish and *C. elegans* for the purpose of imaging. At the same

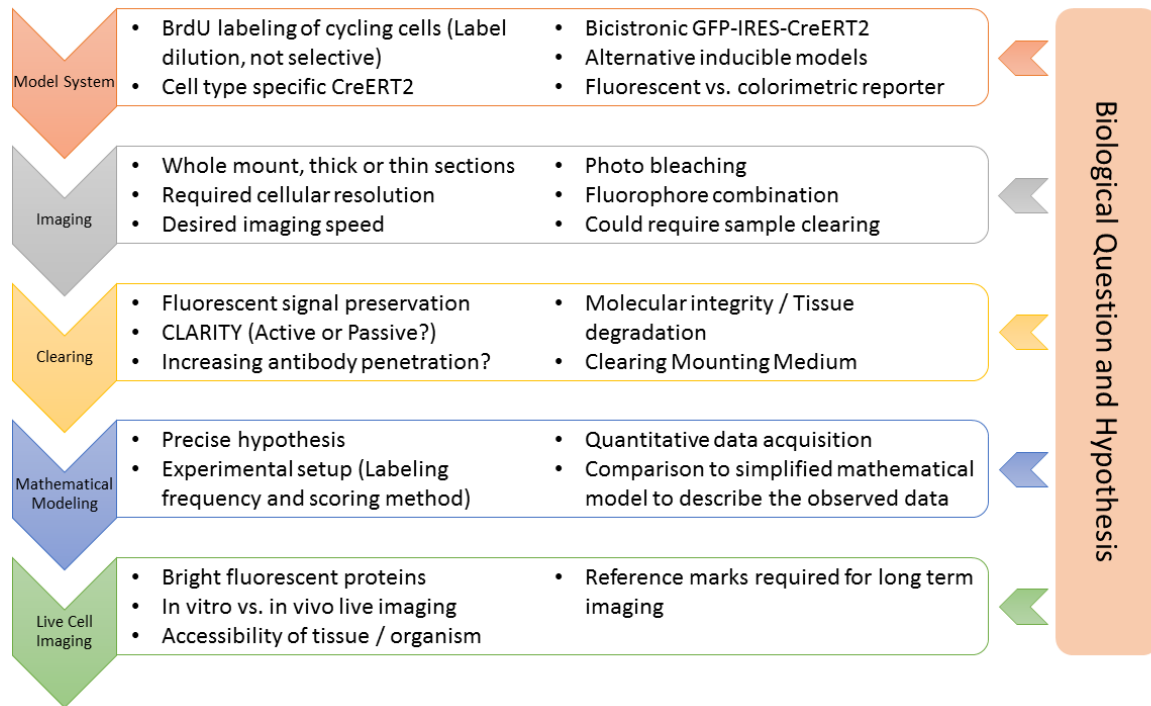


Fig. 5. Overview of available strategies. Lineage tracing experimental design workflow highlighting specific aspects to take into consideration when following a subset or all of the proposed strategies.

time, the lack of transparency is a significant hurdle that obscures vision and imaging of biological processes in many other organisms. Tissue clarification techniques like CLARITY can render some tissue samples transparent and thereby improve imaging conditions (58). However, this procedure cannot be performed on live animals, so while we await the generation of a live transparent mouse it might be interesting to consider model organisms that are naturally transparent. Glass frogs belonging to the family *Centrolenidae* are, potentially, the highest vertebrate displaying high level *in vivo* transparency (131). In combination with CRISPR/Cas genome engineering, they might become a valuable model for genetic and live imaging studies to understand tissue homeostasis (132, 133).

In the end there will, most likely, never be one single ideal approach. Instead, each research question will require its own optimization with regards to model system (*in vitro* or *in vivo*), labeling approach, tissue clearing, image acquisition and relevant mathematical modelling strategies (See Fig. 5 for overview and summary). Together with recent developments and upcoming new systems, lineage tracing and live imaging will provide us with greater details of adult stem cell behavior and tissue homeostasis.

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