

REVIEW

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Intravital microscopy imaging of kidney injury and regeneration



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Abstract

Acute kidney injury (AKI) is a common clinical symptom, which is mainly manifested by elevated serum creatinine and blood urea nitrogen levels. When AKI is not repaired in time, the patient is prone to develop chronic kidney disease (CKD). The kidney is composed of more than 30 different cells, and its structure is complex. It is extremely challenging to understand the lineage relationships and cell fate of these cells in the process of kidney injury and regeneration. Since the 20th century, lineage tracing technology has provided an important mean for studying organ development, tissue damage repair, and the differentiation and fate of single cells. However, traditional lineage tracing methods rely on sacrificing animals to make tissue slices and then take snapshots with conventional imaging tools to obtain interesting information. This method cannot achieve dynamic and continuous monitoring of cell actions on living animals. As a kind of intravital microscopy (IVM), two-photon microscopy (TPM) has successfully solved the above problems. Because TPM has the ability to penetrate deep tissues and can achieve imaging at the single cell level, lineage tracing technology with TPM is gradually becoming popular. In this review, we provided the key technical elements of lineage tracing, and how to use intravital imaging technology to visualize and quantify the fate of renal cells.

Keywords: Lineage tracing, Intravital microscopy (IVM), Intravital imaging, Two photon microscopy (TPM), Kidney, Stem cells

Introduction

The kidney is an extremely complex heterogeneous organ, composed of an organized vascular system and well-defined epithelial components, which are highly coordinated to maintain normal kidney function [1–3]. It is a large challenge to understand the lineage relationship between these cells and the fate map. Furthermore, owing to the technological limitation, observing the behavior of cells *in vivo* is always out of reach, leaving the mechanism underlying renal tissue regeneration invisible. The use of genetically modified mice for lineage tracing offers a forceful instrument for tracking cell dynamics *in vivo* [4]. Previously, lineage tracing was

mainly used in developmental biology, but now it is also applied to stem cell research as it can provide information about the number, location, and differentiation state of progenies of interest cells [5] and their behavior in the intact kidneys [6–9], leading to an increasing attention from researchers across a wide range of scientific disciplines.

Although lineage tracing techniques provide us with a lot of information about tissue development and regeneration, how to accurately interpret the results remains to be solved [10, 11]. Advances in tracking or tracing approaches from light microscopy to two-photon microscopy (TPM), combined with lineage tracing strategies, have allowed researchers to decipher the cellular behavior and fate of various biological processes in the kidney [12]. Owing to TPM can directly visualize the dynamic changes of tissue morphology at subcellular resolution

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in real time in live animals, TPM imaging becomes the preferred experimental technique for studying kidney physiology and diseases over time [13].

Here, we will describe genetic lineage tracing technologies and novel intravital imaging approaches, aiming to outline the existing technical strategies and to elaborate the application of these technologies in kidney development, injury, and regeneration as well as future development prospects. Specifically, we summarize the basic principles and advantages of TPM and the application examples in kidney live imaging technology.

Lineage tracing technology

Cell tracking is a relatively general concept, which refers to labeling and tracking cells. The exploration of cell tracing technology originated in the early 20th century. As early as 1905, Conklin et al. used the characteristics of the coloring difference of the early split bulbs of ascidian embryos to observe the splitting process of split bulbs [14]. Since then, researchers have tried to use water-soluble dyes, fat-soluble dyes, peroxidase, and fluorescent dyes, etc., to physically inject them into cells to track them. Although these technical methods solved some of the problems at the time, there are still many defects in the present view. First of all, the physical method cannot guarantee the accurate labeling of target cells. Secondly, the labeling dye will be gradually diluted until it disappears during the cell division process, and traditional labeling methods cannot effectively permanently label individual cells and their progeny cells. However, due to the backwardness of microscopy equipment at that time, researchers were unable to clearly and continuously track the entire process of cell division [12, 15].

With the maturity of genetic engineering technology, genetic lineage tracing using gene targeting technology has gradually developed. In the application of tracer markers, endogenous genetic markers are used instead of exogenous markers. This type of labeling occurs at the genetic level, enabling long-term labeling of cells and permanent tracing of all their progeny. Lineage tracing techniques require in vivo labeling of specific tissues and cell types at specific times, and site-specific recombinase (SSR) systems with gene targeting technology can meet this need. The SSR has two main members: the Cre (Cyclization Recombination Enzyme)/Loxp (locus of X over P1) recombinase system from *Escherichia coli* phage P1 [16, 17] and the FLP (Flippase)/FRT (Flippase recognition target) system from *Saccharomyces cerevisiae* [18]. The two systems are similar in principle and both cut and connect DNA at specific sites. However, the Cre/Loxp recombinase system has higher recombination efficiency than the FLP/FRT system.

Cre/Loxp recombinase system

The concept of genetic lineage tracing relies on a switch, which is usually a drug-regulated Cre recombinase, which activates the downstream reporter gene. Cre was found in *bacteriophage P1* by Sternberg et al., which recognizes a specific 34 bp nucleotide sequence known as Loxp [19, 20]. Generally, Cre mouse line is hybridized with reporter mice carrying fluorescent proteins or enzymes in a ubiquitous promoter, such as the Rosa26 locus [21, 22]. The expression of the reporter genes is blocked by a strong transcriptional stop sequence flanked by two Loxp sites [22]. After the Cre recombinase is activated, the stop cassette is excised, turning the reporter ON in a way specific to the cell type. On condition that the deletion of the stop sequence is permanent, the reporter gene will be expressed in all descendants of that cell [6, 23–25].

Compared with conventional cell tracking methods, the Cre/Loxp system has a good targeting ability and can only occur in cells that express tissue-specific promoters, largely avoiding the problem of mislabeling. The modified genome and genetic characteristics are the biggest advantages of this system. During development, Cre-expressing cells and all their descendants will be permanently labeled.

Taking advantage of the widely used Cre/Loxp system, the researchers designed a genetic strategy called “Brainbow” [26, 27]. This Brainbow transgenic mouse can randomly express a variety of fluorescent proteins (XFPs) under Cre-mediated recombination [27, 28]. The first generation of brainbow mice (Brainbow-1) used Cre-mediated excision of fragments between Loxp sites to induce recombination events [27]. In Brainbow-2, Cre reverses the DNA fragments defined by the LoxP site and connects them in opposite directions to produce multiple recombination results [27, 28]. The R26R-Confetti mice were created to study the fate map of intestinal stem cells [9]. Clevers and colleagues combined the original brainbow2.1 cassette with the strong CAGG promoter and the LoxP site at Rosa26. After Cre recombination, this R26R-confetti heterozygous mouse showed random recombination of four different fluorescent proteins (nGFP, YFP, RFP, or mCFP) [9, 10]. The ‘Brainbow’, ‘Rainbow’, or ‘Confetti’ mouse models have become a powerful instrument for lineage tracing and has been developed for this purpose [22]. Under the control of a specific promoter, a multicolor reporter gene that randomly generates a large number of fluorescent protein co-expression has been confirmed to be a strategy for dynamic analysis of cell fate and tissue generation. Elena used it will induce this strategy, Bowman et al. used Rosa26-mT/mG mice and Axin2CreERT2 mice to demonstrate the important role of Wnt/ β -catenin signaling pathway in neural stem cell function and

homeostasis throughout the developmental process [29]. Lazzeri et al. tracked tubule cells in conditioned Pax8/Confetti mice and showed that despite the massive loss of renal tubule cells, renal function was restored after AKI. A small portion of Pax2⁺ tubular progenitor cells expand under the injury stress state and regenerate the necrotic tubules [30].

However, this feature also made it impossible to control the labeling time. To address the time specificity of labeling, Metzger et al. created a new mouse model based on Cre/Loxp [31]. This model takes advantage of the nature of estrogen receptors as nuclear receptors, thereby could regulate the time of Cre in the nucleus. Cre fused to a modified form of human estrogen receptor (CreERT) that does not bind estradiol but has affinity to tamoxifen. In the absence of tamoxifen, Cre cannot enter the nucleus due to estrogen receptors remaining in

the cytoplasm, instead Cre enters the nucleus via estrogen receptors only under the condition of exogenous tamoxifen stimuli, accomplishing the recombinant modification of Loxp fragments [32–34]. Therefore, tamoxifen delivery can achieve the time specificity of the knockout event (Fig. 1a).

Lineage tracing in kidney

The use of multicolor reporters can realize the labeling of multiple cell lineages in a specific tissue, so that different types of cells and their progeny can be easily distinguished and imaged by the difference of colors [37]. Recently, combined with time-controlled CreER lines and fluorescent reporter mice, lineage tracing studies have uncovered potential particulars and mechanisms in kidney development, homeostasis, and disease. Specifically, this technique is often used to study several

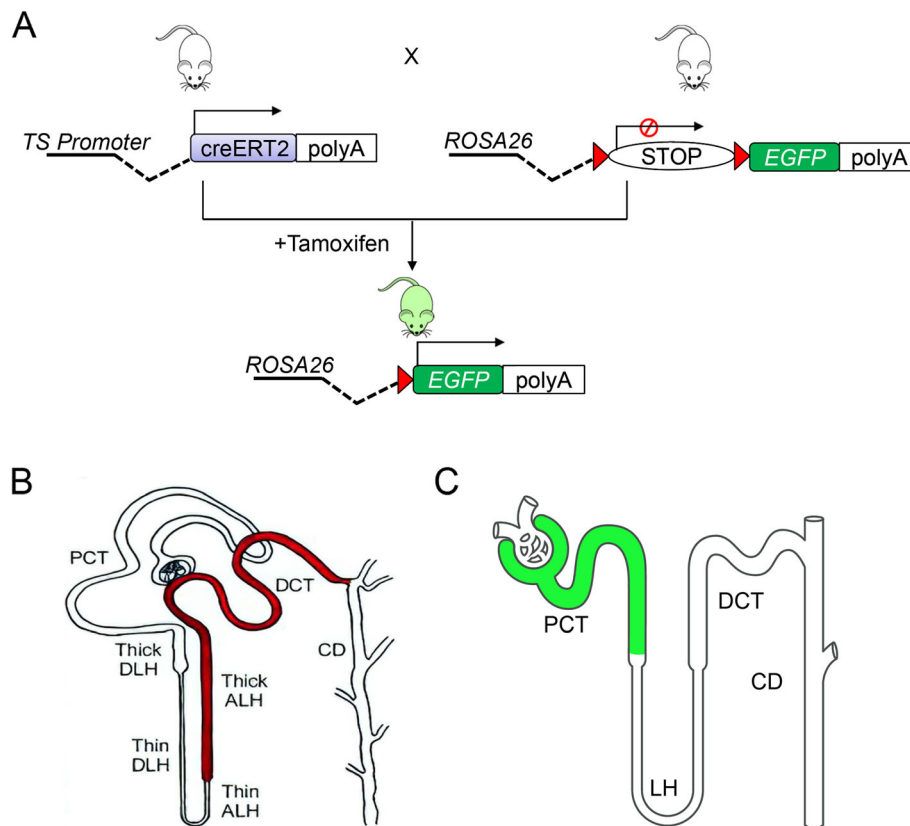


Fig. 1 Lineage tracing of $Lgr5^+$ or $Sox9^+$ nephron stem/progenitor cells using the lacZ or GFP inducible genetic reporter system. **a** First, two transgenic mice were constructed, one was inserted with the Cre gene after the cell type or tissue-specific (TS) promoter, and the other was inserted with a reporter gene, such as GFP, after the promoter of ROSA26, and the expression of the reporter gene was stopped by the STOP sequence flanked by Loxp sites. When the mice of the two transgenic lines are crossed, the Cre enzyme will excise the STOP sequence and the reporter gene GFP will be successfully expressed in a specific cell or tissue [4]. By fusing the Cre gene to the tamoxifen-reactive hormone-binding region of the estrogen receptor (ER), a temporal restriction of expression can be achieved. In the absence of tamoxifen (TAM), Cre is inactive. After administration of tamoxifen, Cre is activated, transported from the cytoplasm to the nucleus, and mediates recombination between Loxp sites. As a result, the STOP codon is excised and the cells are permanently labeled with the reporter gene. The red arrows represent Loxp sites. **b** Schematic diagram of the nephron structure derived from $Lgr5^+$ cells [35]. **c** Graphic abstract of the nephron structure derived from $Sox9^+$ positive cells [36]. Reproduced with permission from Ref. [35, 36]

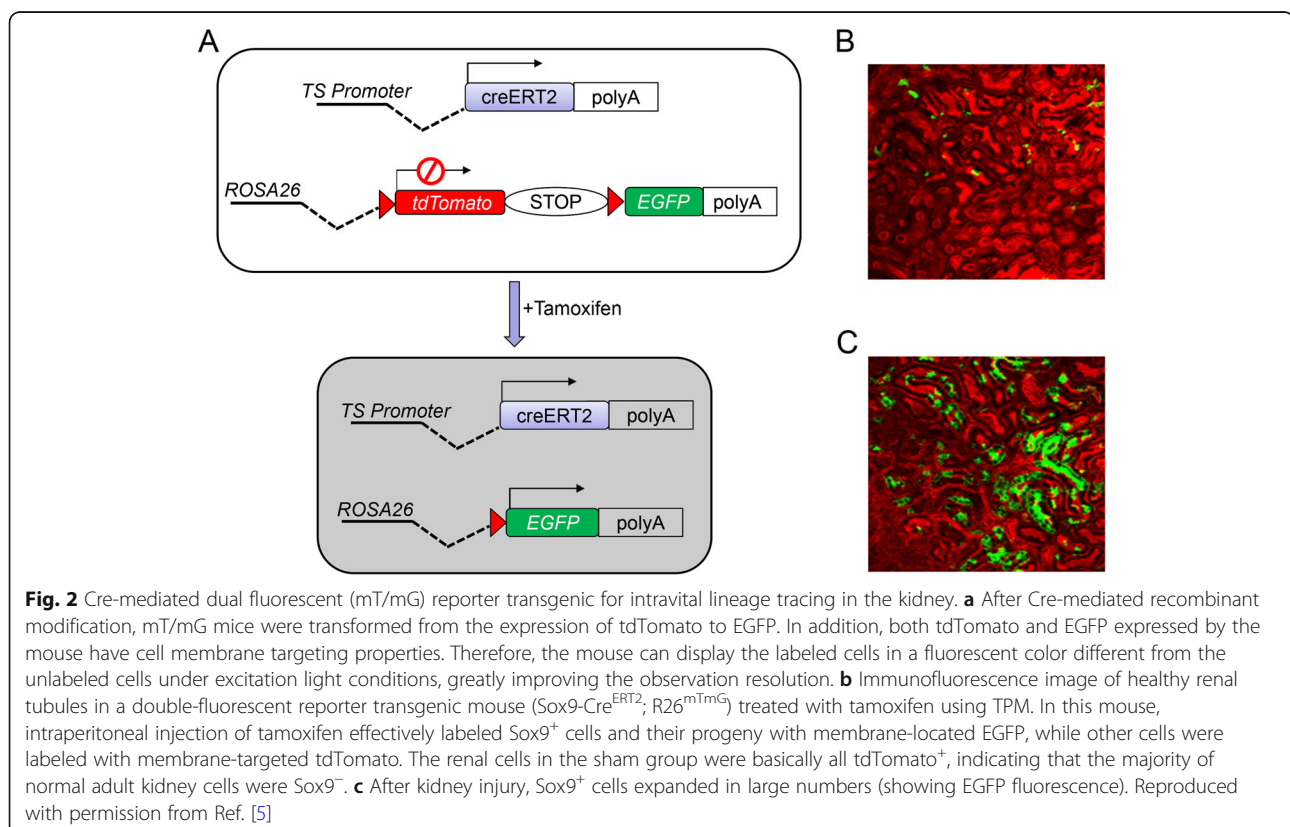
developmental or injury-related renal progenitor cell populations and their lineages, including glomerular epithelial cells (e.g., Pax2 lineage [38]), proximal tubular cells (e.g., Sox9 [5, 36, 39] and SLC34a1 [40]), distal nephrons (e.g., Lgr5 [35]), and collecting ducts (e.g., p63 [41]). Below, we briefly introduce a few typical examples of kidney lineage tracing.

A good example is the research of Lgr5-positive stem cells in the kidney. Muscle, placenta, spinal cord, and other tissues express Lgr5. More specifically, in the intestine, kidney, stomach, ear, hair follicle, and liver, Lgr5 is considered a biomarker of adult stem cells [42]. In the year of 2012, Barker et al. used genetic lineage tracing technology to prove that during kidney development, Lgr5-positive cells are defined as progenitor cells in the kidney, which can develop into distal convoluted tubules and thick ascending limbs of Henle's loop [35, 43]. Specifically, they crossed the Lgr5-EGFP-IRES-CreERT2 mouse lineage with Rosa26-LacZ reporter mice and gave tamoxifen at P1 to activate the Cre enzyme [35]. After Cre is activated, it enters the nucleus to initiate the expression of the lacZ reporter gene in cells expressing Lgr5 in the kidney [35]. Through lineage tracing, they observed that 1 week after the injection of tamoxifen, the progeny cells differentiated from lacZ-positive cells could proliferate rapidly to form a tubular structure [35]. Additionally, to explore the contribution of Lgr5-

positive cells to the structure of the kidney during the development of the mouse kidney, they conducted a year-long induction [35]. Collectively, all these findings demonstrate that Lgr5, as the target of the Wnt pathway, is a biomarker gene of a nephron stem/progenitor population. It contributes to regenerate the thick ascending limb of Henle's loop and distal convoluted tubules in the developing kidney [35] (Fig. 1b).

Another important application of genetic lineage tracing is to determine the epithelial hierarchy of the kidney during AKI and CKD. Sox9 is a transcription factor belonging to the Y box family of sex-determining regions and plays a pivotal role in the development of various tissues and organs including the kidney [44–46]. Studies have shown that mutations in Sox8 and Sox9 cause serious renal dysplasia in mice [36, 39]. In a recent study, researchers took advantage of Sox9-CreERT2 transgenic mice to confirm stem/progenitor cells in the kidney [36]. After kidney injury, Sox9-positive cells are activated and can repair damaged proximal renal tubules (Fig. 2c). In summary, the Sox9-expressing cells isolated from kidney tissue exhibited strong rapid expansion ability and progenitor-like ability in vitro. In vivo lineage tracing experiments showed that Sox9-positive cells were involved in epithelial regeneration after injury [36].

At present, researchers designed a double-fluorescent reporter mouse, which is the membrane-targeted



tdTomato/membrane-targeted EGFP (mT/mG) [47]. In this reporter mouse model, Tomato Red protein is constitutively expressed in all cells in the mouse. The gene segment encoding mT is floxed by two Loxp sites; only cells that experience Cre-mediated recombination activity will express EGFP protein that situates downstream of the mT sequence, thus converting red fluorescence into green (Fig. 2a). In addition, both tdTomato and EGFP expressed in this mouse have cell membrane targeting properties; the labeled cells can be imaged under excitation light, distinguished from the unlabeled ones, responding to a higher resolution. In this way, researchers can verify whether the cells of interest express the reporter allele [47]. The mT/mG reporter system was introduced in the process of renal podocyte turnover and regeneration under three different conditions: aging, unilateral nephrectomy, and acute podocyte loss, respectively. To study the serious loss of glomerular podocytes, Wanner et al. designed a quadruple transgenic model in which mT/mG is located behind the Rosa26 promoter. After injection of doxycycline, it will induce a specific deletion of GFP, thereby marking the newborn podocytes in red [4, 48, 49].

Another mTmG application example is the kidney lineage tracing study from our laboratory [5]. We prepared a kind of mice induced by tamoxifen, named Sox9-Cre^{ERT2}; by hybridizing CreERT2 mice driven by Sox9 promoter with R26^{mTmG} mice, Sox9⁺ cells were specifically labeled. In these mice, intraperitoneal injection of tamoxifen can effectively label Sox9-positive cells and their progeny in green (GFP), while other cells are labeled in red (tdTomato) (Fig. 2b). We found that in normal mice, most of the cells showed tdTomato⁺; when AKI was induced, EGFP-labeled Sox9-positive cells expanded in large numbers, promoting the repair of renal tubular injury (Fig. 2c).

Researchers proposed that using Cre mice constructed with cell/tissue-specific promoters are able to analyze the contribution of potential intratubular stem/progenitor cells to tubular regeneration. SLC34a1 is a sodium-dependent inorganic phosphate transporter, which is only expressed in the differentiated proximal tubule. The researchers used it as a marker for tracking terminally differentiated proximal tubular cells. By integration between lineage tracing and the SLC34a1 promoter, Kusaba et al. concluded that progenitor cells did not contribute to the regeneration of tubular cells after injury, because the fluorescent protein markers were not diluted during the damage repair process [40]. This research depends on the use of cell markers to uniformly label cells and their progeny; however, it does not consider that the intrinsic progenitor cells of the kidney also express differentiated tubular cell markers to a certain extent. Of course, it has also been reported that tissue-

specific progenitor cells express differentiation-related markers in several organs [50–52]. Benjamin D. Humphreys' research team used lineage tracing technology to prove that the regeneration of surviving renal tubular epithelial cells is the main repair mechanism after adult mammalian kidney ischemia-reperfusion injury [53, 54]. As for renal fibrosis, their laboratory uses lineage tracing methods to show that myofibroblasts are not produced by tubular epithelial cells through epithelial to mesenchymal transition (EMT) but derived from endogenous pericytes and interstitial fibroblasts [55]. These studies illustrated well that lineage tracing can be used as a powerful means for exploring the mechanisms of kidney development, disease, and regeneration processes.

Intravital microscopy (IVM)

The structure of the kidney tissue is complex, with a highly branched epithelial morphology, and complicated three-dimensional (3D) imaging of thick tissue is required to analyze the entire fate map of the kidney. In traditional confocal microscopy, a selective filter only allows light of a specific wavelength to pass through, and a pinhole in front of the detector blocks any out-of-focus light, thereby generating an emission image corresponding to the excited sample point. However, confocal microscopy has limitations in photobleaching, excitation light scattering, emission light scattering, and imaging time [56–58].

Different from single photon excitation using continuous photon flux, the basic principle of two-photon excitation is that, at high photon densities, fluorescent molecules can absorb two long-wavelength photons simultaneously, and emit a short-wavelength photon after a short period of time, the so-called excited state lifetime [59]. The effect is equivalent to using a photon having a wavelength of half the long wavelength to excite the fluorescent molecule [58, 60, 61] (Fig. 3a). When two incident photons coincide spatially and temporally, the simultaneous absorption of a single photon results in a selective increase of photon energy, which then obtains the energy required to excite the fluorescent dye or protein of interest. In the case of two-photon excitation, mild infrared or near-infrared light, such as a 750-nm laser, can be used to obtain 450-nm fluorescence. Here, we also list some common fluorescent molecules that can be excited by two photons [57, 62–65] (Table 1). Compared with ordinary laser microscopy, TPM has the advantages of strong tissue penetrating power, high fluorescence signal-to-noise ratio, and small phototoxicity [66–69] (Table 2). It should be emphasized that the kidney is one of the most optically challenging organs for intravital microscopy imaging due to its strong autofluorescence, which is unfortunate for kidney research [70]. Compared with other tissues, such as neocortex,

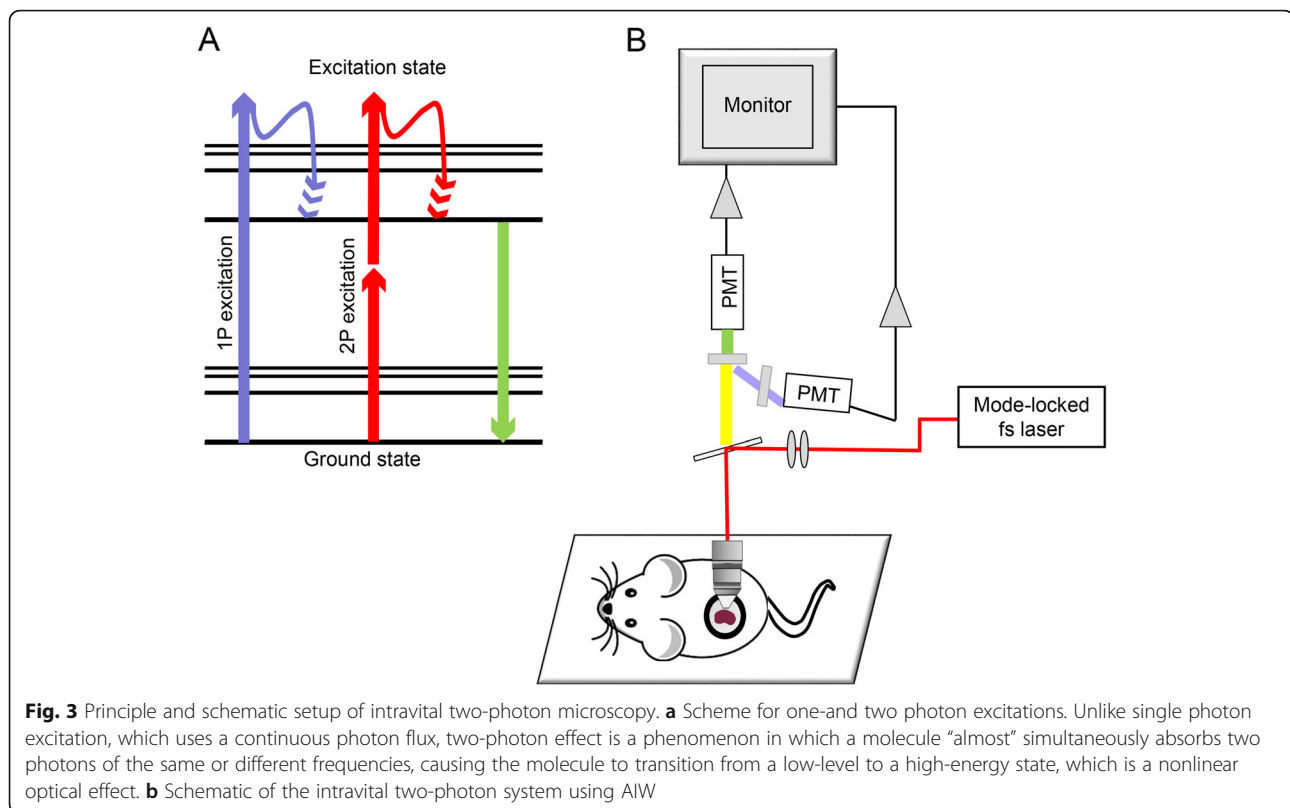


Fig. 3 Principle and schematic setup of intravital two-photon microscopy. **a** Scheme for one- and two-photon excitations. Unlike single-photon excitation, which uses a continuous photon flux, two-photon excitation is a phenomenon in which a molecule “almost” simultaneously absorbs two photons of the same or different frequencies, causing the molecule to transition from a low-level to a high-energy state, which is a nonlinear optical effect. **b** Schematic of the intravital two-photon system using AIW

the depth of intravital TPM imaging can reach 1 mm, while the imaging depth of kidney tissue is about 200 μm , which is far superior to traditional microscopes [63, 71].

The innovation of the abdominal imaging window (AIW) makes the imaging depth of the TPM deeper and can perform kidney intravital imaging over multiple days. This window is composed of a reusable titanium ring, with a 1-mm groove on the side, and a glass slide attached to the top, which is sutured with a purse string to make it tightly fixed on the skin and abdominal wall [72]. The purse-string suture is located in the annular groove and does not directly expose the abdominal cavity, so there is no risk of infection [72–75] (Fig. 3b).

For kidney AIW, the specific surgical procedures are as follows: First, make a 1-cm-long dorsal abdominal incision above the left kidney. Use non-absorbable surgical sutures to connect the skin to the muscular layer of the abdominal wall by purse string suture. Then, we use a 10- μL pipette tip to apply cyanoacrylate glue to the inner surface of the AIW, where the coverslip is attached to the titanium ring. Use forceps to carefully place the AIW on the exposed kidney surface and hold for 5 min until the glue is dry to ensure proper implantation of the window. After AIW implantation, place the abdominal wall and muscles in the titanium ring groove, and carefully tighten both ends of the purse-string suture to fix

the window in place. Tie a double knot at the end of the purse-string suture and then hide the knotted end of the suture in the titanium ring to ensure that the mouse will not lose the suture knot. Allow the mouse to recover on the heating pad for approximately 30 min, and then return it to the cage for postoperative monitoring [76–78]. Under normal circumstances, AIW can last about 2 weeks in mice. After recovery from surgery, AIW serves as a temporary window for observing the live kidney of mice, allowing the study of structural and functional changes during kidney injury and regeneration.

IVM technologies improve the efficiency of kidney lineage tracing studies

The sensitivity and accuracy of fluorescent reporters continue to increase, making genetic recombination very suitable for live imaging of living cells. Today, primary technical progress in the evolution of microscopy (e.g., two-photon microscopy) combined with genetically encoded fluorescent proteins of cell lineage enables intravital imaging technology to be used for monitoring biological events in real time in living animals [79–81]. Thus, at present, intravital microscopy (IVM) largely relies on the detection of fluorescence signals and it has helped to track the fate and function of single renal cells (Table 3).

Table 1 Some common fluorescent molecules for two-photon excitation [57, 62–65]

Application	Fluorophores	2P excitation (nm)	Emission (nm)
Membrane probe	Dil	830~920	565
	DiO	780~830	510
	Filipin	720	510
Nucleic acid stains	Hoechst	780~820	455, 478
	PI	820~850	617
Mitochondrion probe	Mito Tracker red	750~840	600
Fluorescent proteins	eYFP	860~950	532
	eGFP	820~950	509
	eCFP	800~900	476
	eBFP	800	445
	tdTomato	760~800	581
	mCherry	750	610
Conjugates dyes	CY2	780~800	506
	CY3	780	565, 615
	CY5	780~820	670
	FITC	740~820	519
	Rhodamine 123	780~860	550
Conjugates dyes	Alexa 350	750~800	440
	Alexa 488	720~800	515
	Alexa 516	720~840	569
	Alexa 568	720~840	596
	Alexa 594	720~860	580
	Alexa 633	720~900	647
	bis-MSB	680~750	420
	Neural tracer	Bodipy	900~950
	DiD	780~820	670
	Lucifer Yellow	860~890	533

Dil 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, *DiO* 3,3'-dioctadecyloxycarbocyanine perchlorate, *PI* propidium iodide, *YFP* yellow fluorescent protein, *GFP* green fluorescent protein, *CFP* cyan fluorescent protein, *BFP* blue fluorescent protein, *FITC* fluorescein isothiocyanate, *DiD* 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine,4-Chlorobenzenesulfonate salt

Table 2 Comparison of several fluorescence microscopy in imaging [12]

Property	Two-photon microscopy	Standard fluorescence microscopy	Laser scanning confocal microscopy
Imaging depth	< 1000 μm	All (out of focus)	< 500 μm
Effective resolution	High	Low	Low
Photo bleaching	Low	Medium~high	Medium
Excitation	Limited to objective focal volume	All	Entire specimen in objective focal axis
Emission	Dependent on specimen and image depth	N/A	Dependent on specimen and image depth
Main limitations	Imaging time, heat, price	Imaging depth, Z-resolution	Imaging time, photo damage
Main advantages	Imaging depth, low photodamage	Accessibility very good for 2D of thin sections, additional deconvolution	Accessibility, 3D imaging
Cost	Very high	Low~high	High

Table 3 Imaging technologies promote tissue-wide lineage tracing studies

Imaging technologies	Transgenic mice	Outcome	Ref/
Bioluminescence imaging (BLI)	<i>Ngal-Luc</i>	Illuminated injuries in vivo in real time	[82]
Multiphoton microscopy (MPM)	Podocin-GFP; Podocin-confetti; PEPCK-GFP	Visualize the motility of podocytes and parietal epithelial cells (PEC) in vivo	[83]
Confocal microscopy	Coll1 α -GFP-CreERT2; R26LacZ; R26mTmG; R26Tomato	Easily detect anatomic features of podocytes	[84]
Multiphoton microscopy (MPM)	Pod/Cre-GCaMP3/fl	Reveal the importance of podocytes Ca ²⁺ in glomerular pathology	[85]
Two photon microscopy (TPM)	Confetti/Podo:Cre; CA-Rac1/Nphs1-rtTA	Podocytes change from a static to a dynamic state in vivo	[86]
Two photon microscopy (TPM)	GFP-CaMP2	Detect basic calcium levels in proximal tubular epithelial cells	[87]
Multiphoton microscopy (MPM)	PDGFR β -CreERT2; R26-mTmG	PDGFR β mediates the communication between the renal interstitium and the tubule system	[76]
Two photon microscopy (TPM)	Sox9-CreERT2; R26-mTmG	In vivo imaging of the fate of green Sox9 ⁺ cells	[5]
Two photon microscopy (TPM)	mRen-Cre; R26-mTmG	The niche of progenitor cells of the renin lineage cell is continuously filled by the neonatal differentiation of the kidney	[88]
Multiphoton microscopy (MPM)	Ren1c-Cre; R26-Confetti; Ren1d-Cre; R26-Confetti	In focal segmental glomerulosclerosis, renin lineage cells migrate to the glomerulus and replace podocytes and PECs	[89]

Scientists have utilized the characteristics of firefly luciferase and Renilla luciferase to perform bioluminescence imaging (BLI) in the presence of specific substrates to develop a reporter system that allows non-invasive tracking of cells [90, 91]. For example, the Barasch group fused the *Ngal* gene, a marker of kidney damage, with the luciferase reporter gene to generate *Ngal-Luc* reporter mice. In this mouse model, when kidney damage occurs, the expression of the *Ngal* gene is induced, thereby activating luciferase expression, reproducing the endogenous information, and illuminating damage in vivo in real time [82].

The above strategy was different from the Cre/Loxp system. Although BLI has a small background and high signal-to-noise ratio, it cannot provide information on cell resolution [92]. This problem can be circumvented by using intravital microscopy (e.g., confocal TPM) combined with fluorescent reporter transgenic animal models. It is not surprising that this was mainly established in zebrafish and mouse models, which have the most advanced and comprehensive genetically modified strains [70].

Podocytes together with vascular endothelial cells and glomerular basement membrane constitute the glomerular blood filtration barrier. The special anatomical location of podocytes makes it difficult to study in vivo. The *János Peti-Peterdi* group used multiphoton microscopy (MPM) to track the lineage development of the same glomerulus over several consecutive days to visualize the movement of podocytes and parietal epithelial cells

(PEC) in vivo. The transgenic mice they used were Podocin-GFP mice, Podocin-confetti mice, and PEPCK-GFP mice. Their results show that the glomerular environment and cell composition are highly dynamic rather than static. MPM intravital imaging combined with genetic lineage tracing is expected to promote the understanding of glomerular injury and regeneration mechanisms [83]. Similarly, the *Benjamin D. Humphreys* laboratory utilized a multicolor labeling method to track the changes in podocyte morphology after injury over time, which were performed by conventional fluorescence (optical) microscopy in fixed kidneys and ex vivo [84].

Intravital microscopy imaging can also observe the inside of a single cell of the intact living kidney, and quantitatively display intracellular variables and cell signals. Researchers combined genetic lineage tracing techniques with intravital imaging technologies to study Ca²⁺ signals in the nephron. Burford et al. conducted an experiment, they developed an imaging approach that used TPM to directly visualize podocyte Ca²⁺ dynamics within the intact kidneys of live mice expressing a fluorescent calcium indicator only in podocytes [85]. They designed a new podocin/Cre-GCaMP3/flox mouse model and combined with MPM, so that they can directly visualize Ca²⁺ dynamics in the critical but inaccessible renal cell type of podocytes in vivo in the intact kidney [85]. For the first time, they combined intravital TPM imaging with transgenic mice for research. This method can be a powerful tool for detecting changes in

podocyte Ca^{2+} in glomerular function in physical or disease states. In *Kornelia Szebenyi* group, they used a calcium-sensitive fluorescent probe to provide the first detailed description of intracellular calcium signals in kidney tubules in vivo. They produced a transgenic rat stably expressing the GFP-calmodulin based genetically encoded calcium indicator (GCaMP2) predominantly in the kidney proximal tubule. They used in vitro confocal microscopy and in vivo TPM to detect basic calcium levels in proximal tubular epithelial cells, as well as changes in these levels caused by ligands and drugs [87].

In glomerular disease, podocyte damage leads to large changes in cell morphology. However, whether podocytes are static or actively moving cells in the body is still controversial. To analyze the motility of podocytes in vivo, the researchers utilized intravital TPM to image the kidneys of Confetti mice in vivo. They discovered that uninjured podocytes stay inactive, while transfer into a dynamic state after suffering from injury [86].

To better illuminate the potential role of interstitial cells in renal tubular regeneration, the laboratory of *Ina Maria Schiessl* recently used AIW to trace PDGFR β -mTmG transgenic mice via intravital MPM. This research provided visual evidence that renal interstitial cells play a crucial role in renal tubular epithelial regeneration [76].

Another outstanding example of the application of intravital imaging technology via AIW to enhance the efficiency of kidney lineage tracing comes from our research group. We utilized the AIW to perform intravital imaging of Sox9-positive cells under TPM. Using the high resolution of AIW, we performed TPM intravital imaging with a deepness of 200 μm and observed the kidney structure. We performed serial 8 real-time imaging on days 1, 3, 7, and 14 after ischemia-reperfusion injury to continuously observe the proliferation of Sox9-positive cells. By combining kidney Sox9 lineage tracing with two-photon intravital imaging technology, we can intuitively observe the kidney injury and regeneration in real time in vivo, greatly improving the understanding of the results of lineage tracing [5].

In summary, the application of genetic lineage tracing techniques in combination with modern intravital imaging will enable long-term lineage tracing studies of kidney development, homeostasis, and disease. However, so far, there are not many studies on the combined application of intravital imaging technology and genetic lineage tracing strategy to the kidney. This may be affected by factors such as the imaging depth of the intravital microscopy, the complex structure of the kidney, and the respiratory rate.

Conclusion

Lineage tracing is an overwhelming strategy that is widely used to interpret the pathophysiological

mechanisms of kidney injury and regeneration and now becomes accessible to nearly all scientists. With the appearance of a large number of novel Cre driver mouse lines, fate tracking of any cell type in the kidney can be achieved. This forceful technology also has limitations, such as the difficulty of interpreting complicated results. Despite all these deficiencies, lineage tracing techniques provide more intuitive and scientific research means for exploring the origin of cells in normal development, disease occurrence, and injury repair processes. Recently, intravital imaging with advanced optical equipment such as TPM has provided a forceful tool for the tracing of live cell lineages. It has been demonstrated that using intravital microscopy can accurately quantify the complex dynamic cellular processes in the kidneys and assess their real-time response in pathological states. With the continuous improvement of laser, optics, and microscopy technologies, the development of new TPM imaging modalities and their combination with transgenic animal models will further promote their use in the study of kidney disease or may be used for clinical diagnosis.

Abbreviations

AKI: Acute kidney injury; BFP: Blue fluorescent protein; BLI: Bioluminescence imaging; CFP: Cyan fluorescent protein; CKD: Chronic kidney disease; Cre: Cyclization Recombination Enzyme; GFP: Green fluorescent protein; IVM: Intravital microscopy; MPM: Multi-photon microscopy; mTmG: Membrane-targeted tdTomato/membrane-targeted EGFP; PEC: Parietal epithelial cells; RFP: Red fluorescent protein; TPM: Two-photon microscopy; YFP: Yellow fluorescent protein

Acknowledgements

Not applicable.

Authors' contributions

YL conceived and wrote the manuscript. ZL revised and approved the final manuscript. The authors read and approved the final manuscript.

Funding

This research was partially supported by the National Key R&D Program of China (2017YFA0103200) and National Natural Science Foundation of China (U2004126).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 14 December 2020 Accepted: 4 May 2021

Published online: 13 May 2021

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