

Characterization of the calcium-actin cytoskeleton axis in glomerular podocytes



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Eva Wiesner

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Reviewers:

Prof. Dr. Thomas Benzing

Prof. Dr. Jan Riemer

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Abstract

Glomerular podocytes are one component of the renal filtration barrier. Many kidney diseases inflict harm on these unique cells, with their loss being irreversible. Foot process effacement is one hallmark of podocyte damage, but so far, it is not known which signals drive the changes in their morphology. Identification of universal response mechanisms to damage could serve as future therapeutic targets for treating podocytopathies and other kidney diseases.

We hypothesize that the calcium-actin axis modulates junctional composition in glomerular podocytes during foot process effacement. Here, we show that podocytes react with a reversible change in morphology to damage, which involves rearrangements of the actin cytoskeleton and slit diaphragm composition. We utilized multiphoton and STED microscopy together with the endogenous fluorescent reporters GCaMP3 and the novel LifeAct.mScarlet-I in *in vivo*, *ex vivo* and fixed tissue imaging approaches. Calcium baseline concentrations increased after nephrotoxic serum induced nephritis. This caused foot process effacement in a non-correlative manner. The effacement was partly reversible as podocytes recovered in later NTS nephritis time points. The actin cytoskeleton formed distinct patterns during effacement, independent of disease origin, that co-occurred with changes in cellular junction protein composition. The slit diaphragm transitioned towards a tight junction by recruiting ZO-1 upon injury, while normal foot process morphology was lost.

Our data contributes to the understanding of different cellular components involved in maintaining podocyte filtration barrier integrity. It not only demonstrates cellular adaptations after podocyte injury but also how they are reversed once the injury is resolved. With a novel fluorescent reporter, we gained an unprecedented view of the slit diaphragm composition during disease. Future studies dissecting the effect of the proteins involved in these processes will provide complementary mechanistic insights into podocyte biology.

Introduction

1.1 The Glomerular filtration barrier and podocyte biology

1.1.1 The glomerulus as the functional filtration unit of the kidney

Mammalian kidneys perform and regulate several biological functions, such as the filtration of blood to excrete waste, regulating blood pressure by maintaining fluid homeostasis and hormonal secretion. An initial volume of up to 180 L primary filtrate can be generated by the glomeruli, the functional units for filtration, per day (Fig. 1). The downstream tubular system reabsorbs fluid and salt, thus concentrating the primary filtrate to about 1 L of urine.

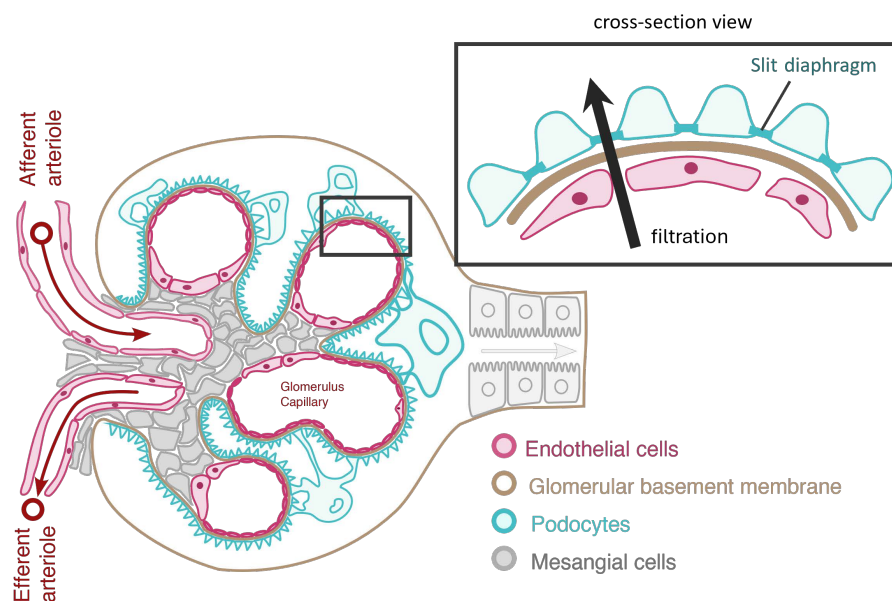


Figure 1: The glomerular filtration barrier. Several cell types are present in the renal glomerulus: mesangial, fenestrated endothelial cells and podocytes. Between two podocytes' foot processes, the slit diaphragm is formed. Blood enters through the afferent arteriole and passes through capillaries, then exits the glomerulus through the efferent arteriole. Blood pressure exerts filtration pressure and liquid passes the filtration barrier consisting of the three layers seen in the cross-sectional view. First, it passes the fenestrated endothelium, then the glomerular basement membrane and ultimately the slit diaphragm formed by podocytes. Modified after [1]

Within the glomerulus, blood enters through the afferent arteriole, passes through convoluted capillary structures and exits the glomerulus through the efferent arteriole. The important properties of the filter are 1) high permeability to water and small molecules and 2) the retention of larger macromolecules such as proteins. The filtration barrier prevents molecules larger than 70kDa from passing into the Bowman's space by a three-layered barrier setup. The first layer is formed by fenestrated endothelial cells covered by a glycocalyx. The second layer consists of the glomerular basement membrane, which is formed by secreted extracellular matrix components, e.g. collagen Type IV. The last layer is formed by glomerular podocytes. During maturation of the glomerulus, they establish a specialized cell-cell junction, the slit diaphragm. Podocytes play a crucial role in maintaining filtration barrier function and therefore, are of special interest for studying and understanding fundamental pathomechanisms in human diseases.

1.1.2 Podocyte biology and function

Podocytes are terminally differentiated, polar epithelial cells. Podocyte progenitors emerge during nephrogenesis in the S-shaped body stage and mature during the capillary loop stage. Ultimately, they lose their mitotic potential, making their loss upon injury a major problem in

kidney disease [2]. Podocytes develop several unique morphological features. Their cell body extends long cellular protrusions, the primary processes that wrap around the capillary loops in the glomerulus. These primary processes branch further into smaller foot processes (FP). These interdigitate and form the previously mentioned cell junction, the slit diaphragm, which is the last layer of filtration and is approximately 40 nm wide [3]. The slit diaphragm is a highly specialized junction containing podocyte-specific proteins needed for scaffolding and signaling. Its protein composition is distinct from adherens or tight junctions, although containing some associated components.

One major slit diaphragm protein is nephrin, encoded by the *NPHS1* gene. Mutations in *NPHS1* cause congenital nephrotic syndrome of the Finnish type [4][5]. Nephrin is a transmembrane protein of around 190kDa. It consists of a short intracellular domain and a larger extracellular domain built of eight IgG-like motifs (Fig. 2) [6]. Its cis- and trans- homophilic interactions with other nephrin molecules expressed on neighboring podocytes FPs are the basis for the cell-cell connection forming the slit diaphragm [7].

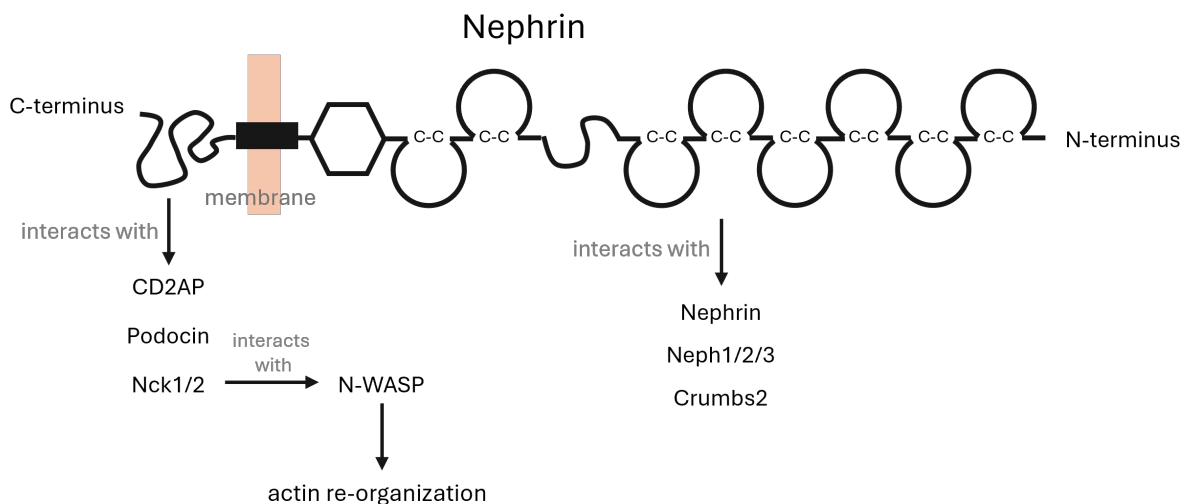


Figure 2: Nephrin structure and interaction at the slit diaphragm. Nephrin consists of eight extracellular C2-type IgG domains interconnected by disulfide bonds, a fibronectin type-III domain and a cytoplasmic tail. The extracellular domain can form cis- and trans- interactions with other nephrin molecules. It can bind other slit diaphragm proteins like Neph1/2/3 or Crumbs2. The intracellular part transduces signals to downstream targets. It can directly bind, e.g., podocin, CD2AP and Nck1/2. Nck1/2 can bind N-WASP and therefore influence actin cytoskeleton organization. Modified after [6] [7].

The extracellular domain interacts with other slit diaphragm proteins like NEPH1-3 or Crumbs2 [8], [9], [10]. The intracellular C-terminal part of nephrin is considered an important signaling hub. It can bind proteins, such as podocin or CD2AP, but also transduces signals by phosphorylation-dependent binding [11], [12]. One known interactor is Nck1/2, with its binding to nephrin, it activates downstream signaling, influencing actin distribution [13][14]. Through its wide range of binding partners, nephrin signaling modulates several cellular processes, such as cell survival, polarity, adhesion and scaffolding processes [15][16][17].

For a long time, the nature of the slit diaphragm as a junction and its involvement in maintaining the filtration barrier have been discussed. It can be seen as a modified adherens junction, as typical adherens junction proteins such as vinculin, catenins and p-cadherin are expressed in podocytes [18]. Their role in maintaining the slit diaphragm integrity varies, as the knockout of β -catenin or vinculin shows no overt phenotype in mice, but their loss impacts podocytes' response to damage [19][20]. The slit diaphragm also contains typical tight junction proteins such as ZO-1 and Claudin-5. Their knockout in podocytes is associated with a loss of protein into the urine

(proteinuria) and a flattening of FP, one hallmark of podocyte injury [21], [22]. It has been proposed that upon podocyte damage, the junctional composition of the slit diaphragm changes to form a more tight junction-like cell contact, to adapt to harmful conditions [23], [24].

1.1.3 Podocytes in glomerular disease

Due to their limited regenerative capacity, podocyte damage and loss are a leading cause for the development of kidney disease. Injury to podocytes leads to filtration barrier failure and results in proteins passing into the urine, so-called proteinuria. A complete loss of podocytes into the urine (podocyturia) results in hypertrophy of remaining cells, infiltration of glomerular parietal epithelial cells and glomerular scarring [25], [26], [27]. Insults to podocytes can stem from three main origins. Firstly, they can be hereditary, caused by mutations in podocyte-specific genes such as *NPHS2* encoding for the protein podocin. Podocyte injury can also be acquired, caused by diseases such as hypertension, diabetes, or initiated by toxicological damage. Lastly, podocytopathies can be caused by immune-mediated damage, mostly resulting in minimal change disease, focal segmental glomerular sclerosis (FSGS) and several forms of nephritis. Recently, the discovery of anti-nephrin antibodies provided a new view on the pathogenesis of minimal change disease, reinforcing an immune-mediated etiology for podocyte damage [28], [29].

As one pattern of glomerular injury, FSGS is characterized by scarring in a fraction of glomeruli and only in segments of their whole area. Clinically, FSGS patients present with proteinuria, edema and renal failure. One hallmark of FSGS is that foot processes lose their unique shape by simplifying, flattening and fusion, a process termed ‘foot process effacement’. While there are a plethora of possible triggers for podocyte injury, they share foot process effacement as a common characteristic. Effacement is associated with abnormal calcium signaling and cytoskeletal changes [30]. The importance of these pathways is underlined by the fact that mutations in genes encoding for the calcium channel TRPC6 or the actin-binding protein α -actinin-4 cause FSGS in humans [31], [32].

Another disease phenotype affecting podocytes is glomerulonephritis. It is characterized by an immune complex mediated response, targeting glomerular structures, especially blood vessels, causing inflammation. This can cause the loss of red blood cells in the urine (hematuria) alone or along with proteinuria. Glomerulonephritis may also present with hypoalbuminemia in the serum, hyperlipidemia and edema. Increased serum creatinine and urea are additional markers for renal insufficiency that might occur during glomerulonephritis.

One way to study glomerulonephritis in rodents is to induce it with nephrotoxic serum (NTS). In 1934, the Japanese researcher M. Masugi established a working experimental model and coined the term ‘masugi nephritis’ [33]. For introducing NTS-mediated nephritis, experimental animals are injected with a polyclonal serum containing antibodies against glomerular structures. To raise those antibodies, another host species, usually rabbit or sheep, is immunized with crude glomerular extracts of the experimental species. The host’s serum contains mostly antibodies against the GBM, but also ones targeting other glomerular structures. Since the preparation of NTS can vary in composition, its toxicity depends on the inflammatory response of the host and target, the dose and the application scheme, causing a varying nephritis severity. NTS nephritis is made up of two main disease phases: the heterologous phase and the autologous phase [34]. In the acute heterologous phase, the serum antibodies are deposited in the glomerulus. Consecutive inflammation is driven mostly by complement activation [35]. During this phase, proteinuria and foot process effacement are present [36]. The autologous phase follows and is dominated by an adaptive immune response against the injected, deposited antibodies. It is

mostly mediated by Th1 and Th17 cells [37]. Initial glomerular damage progresses to crescent formation and glomerulosclerosis through inflammatory processes. Recent studies have shown that abnormal calcium homeostasis is one factor in early NTS nephritis pathogenesis, accentuating the importance of calcium signaling in podocytes [38], [39].

1.2 Calcium as a universal and versatile messenger

1.2.1 General principles of calcium signaling

Calcium can act as a universal messenger for cellular communication and signal transduction. It is involved in muscle contraction, neuronal activity, movement, fertilization and apoptosis [40], [41]. Stimuli for initiating calcium influx can be ligand binding to their respective receptor, like hormones or neurotransmitters. It can also be triggered independently through mechanical stress. Calcium can either act directly on other proteins, like ion channels, for immediate effects or act as a second messenger through the transmission of signals through G protein-coupled receptors. Calcium concentrations are maintained at a low intracellular level (~100 nM), while concentrations for the endoplasmic reticulum (ER) stores and the extracellular space are 20.000 fold higher [42]. The increase of cytoplasmic calcium can therefore originate either from external or internal sources. Ion exchangers and pumps actively uphold the cellular calcium gradient.

Calcium signaling can modulate a plethora of processes by coupling the output to parameters like speed, amplitude and spatio-temporal patterning of the signal [41]. One way to transduce calcium signals is through the phospholipase C (PLC) pathway. Membrane receptors, including G protein-coupled receptors and tyrosine kinases, activate PLC. The activation leads to the consequent hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). DAG modulates protein kinase C activity, while IP₃ binding to ER receptors leads to internal storage calcium release [43]. Calcium can also bind the protein calmodulin and act as a second messenger, thereby regulating Ca²⁺/calmodulin-dependent kinase signaling [44].

Calcium signaling can rapidly trigger cytoskeletal remodeling. The calcium-mediated actin reset (CaAR) described in 2016 is a fast and transient actin reorganization, with actin disassembling at the cellular cortex and reassembling at the ER upon calcium influx [45]. This mechanism is conserved across several cell types and species, including glomerular podocytes in cell culture.

1.2.2 Calcium signaling in podocytes

Abnormal calcium signaling is a shared feature in podocyte injury [38], [39], [46]. The role of transient receptor potential cation channel member 6 (TRPC6) was extensively studied in recent years. Human mutations in the *TRPC6* gene leading to either a gain of function or a loss of function cause FSGS [47], [48]. TRPC6 is located in the podocyte cell body and in proximity to the SD, where it was shown to bind nephrin and podocin [49], [50], [51]. TRPC6 is known to be mechanosensitive and therefore functions as a transducer for stretch-related signals in podocytes due to altered environmental pressure [52], [53]. It was also shown that TRPC6 activates the small GTPase Rac1 that controls actin dynamics [54]. Additionally, TRPC6 was shown to induce actin cytoskeleton disruption through RhoA [53].

The calcium-actin signaling axis is especially important for podocytes during differentiation. In zebrafish larvae, podocytes generate PLC-activity-dependent calcium transients during, but not after, maturation of the filtration barrier [54]. The blocking of this calcium release inhibited the proper formation of FP, highlighting the tight connection between calcium signaling and actin

dynamics. Subsequently, podocyte injury to mature larvae led to re-initiation of calcium transients, suggesting that podocyte injury responses may be mediated through calcium signaling.

1.3 The actin cytoskeleton in podocyte structure and function

1.3.1 Actin architecture in the foot process

Under physiological conditions, podocytes must withstand fluctuating forces exerted by blood flow and urinary filtration into the Bowman space. The adaptability of their cytoskeleton to preserve filter barrier integrity relies heavily on actin remodeling. In general, the podocytes' cytoskeleton differs in each cell compartment. The primary processes' cytoskeleton mostly consists of tubulin and intermediate filaments like vimentin (Fig. 3). The smaller FPs are supported by a cortical actin network and a contractile actin filament apparatus, including non-muscle myosin-IIa and synaptopodin [55], [56], [57].

In nearly all forms of glomerular disease, cytoskeletal changes are present and especially FP effacement involves active reorganization of actin fibers [34], [58]. The flattening of FP is accompanied by the formation of an 'actin mat' described using electron microscopy in 1996 [59].

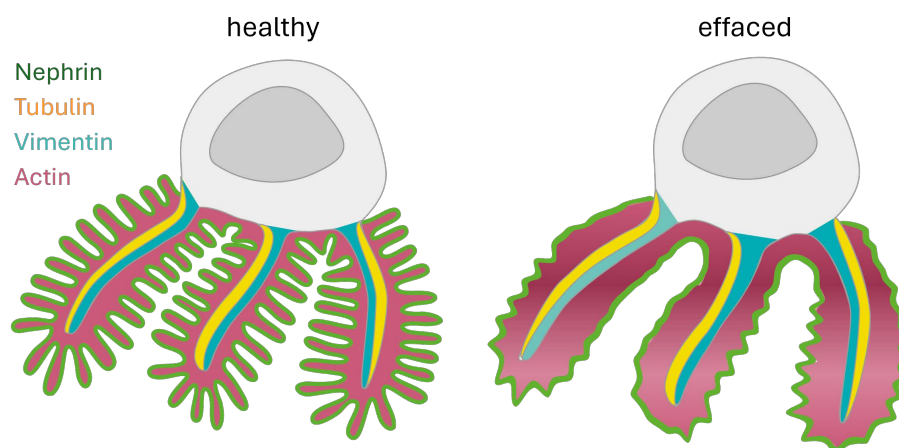


Figure 3: Podocyte cytoskeleton during foot process effacement. In healthy podocytes, the FP form a regular pattern with elongated FPs. After effacement, the FPs' shape is lost and they shorten and lose curvature. The two major cytoskeletal components of the primary process are acetylated tubulin and vimentin. Actin is present mostly in the FPs.

Not only actin dynamics themselves but also actin-binding and regulatory proteins are important and affect the upkeep of the filtration barrier (Fig. 4). For a tight adhesion to the GBM, focal adhesions are formed with $\alpha 3\beta 1$ integrin as their most important component. Focal adhesion kinase further regulates adhesion and motility in podocytes and its phosphorylation influences Rho GTPase activity and thereby actin modulation [60], [61]. Another important actin-binding protein is α -actinin-4. It is responsible for bundling of F-actin fibers and mutations in the *ACTN4* gene cause FSGS, affecting podocytes in the kidney, while no other tissues or organs are clinically affected [30].

The slit diaphragm, as a signaling hub, also influences cytoskeleton dynamics. Nephrin connects the slit diaphragm to the actin network through several signaling cascades and protein interactions. Nephrin is known to bind the scaffolding protein CD2-associated protein (CD2AP), which binds to actin and podocin [62], [63]. CD2AP modulates actin dynamics through a diverse interactome with Rac1, CapZ, cortactin and Synaptopodin [64], [65], [66], [67]. A knockout of CD2AP in mice leads to nephrotic syndrome [68]. Nephrin signaling also regulates actin kinetics.

By phosphorylation of nephrin, Nck is recruited to the slit diaphragm [69]. Nck binding triggers the recruitment of N-WASP, which regulates actin filament nucleation and branching, possibly through the Arp2/3 complex [70], [71].

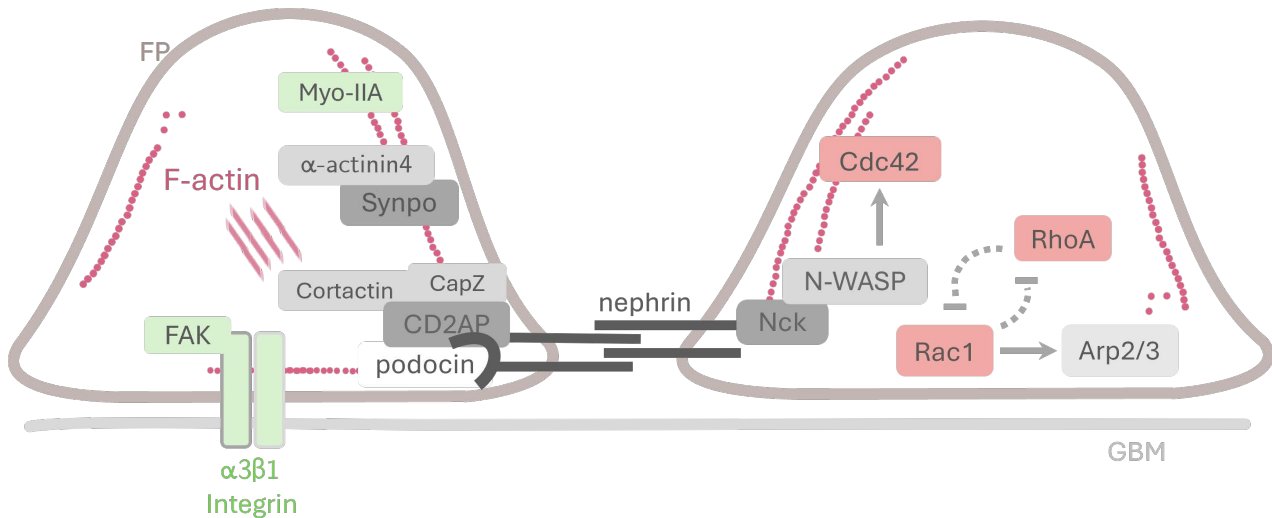


Figure 4: Actin network in podocyte foot processes. Actin fibres (pink) are present at the cortical part of the FP and a central actin bundle runs along the longitudinal axis of the FPs. The slit diaphragm proteins (black), nephrin and podocin, bind to adapter proteins like Synaptopodin (Synpo), CD2AP, or Nck (dark grey). Effector proteins like α -actinin-4, cortactin, CapZ, N-WASP and Arp2/3 (light grey) regulate actin dynamics through several mechanisms, e.g., nucleation, bundling, or capping of actin fibres. Adhesion and motility components (green), like non-muscle myosin IIA (Myo-IIA), are part of the contractile apparatus. Adhesion of the actin cytoskeleton to the GBM is mediated by focal adhesion kinase (FAK) and $\alpha3\beta1$ integrins. The small GTPases (light pink colour) RhoA, Rac1 and Cdc42 regulate the formation of lamellipodia or stress fibres. Modified after [72], [73]

1.3.2 Small GTPases and actin regulation and their role in podocytes

The small Rho family GTPases RhoA, Rac1 and Cdc42 are key regulators of actin dynamics. Their role in balancing cytoskeletal rearrangements in podocytes has been debated for a long time. Only the loss of Cdc42 causes proteinuria and effacement due to ultrastructural abnormalities [74], [75], while Rac1 and RhoA activity affect podocytes differently. Generally, Rac1 promotes the formation of lamellipodia and the upregulation of Rac1 activity in podocytes led to proteinuria and FP effacement, possibly due to an increase in motility [76], [77], [78]. RhoA, on the other hand, is driving stress fiber formation and is a known interactor of Synaptopodin [79]. Although its loss specifically in podocytes shows no related injury phenotype, dysregulated activity can alter the intricate actin homeostasis needed for cellular adaptation [74]. Both dominant negative and constitutive active forms of RhoA cause glomerulosclerosis and proteinuria in mice [80], [81]. Small GTPase activity is also tightly connected to calcium signaling. The TRPC6 ion channel is known to activate RhoA, while the TRPC5 channel is involved in Rac1 regulation [53], [82]. In general, the balance of small GTPases activity plays a crucial role in maintaining actin cytoskeleton integrity in podocytes.

Further studies might elucidate pathways connecting the actin cytoskeleton, focal adhesions and slit diaphragm signaling, as their seamless interaction is mandatory for glomerular filtration barrier integrity.

1.4 Advanced microscopy approaches to study podocyte dynamics

1.4.1 Multiphoton microscopy for intravital imaging

For a long time, light microscopy was limited to thin sections of tissue, one reason being the scattering of light, which limited obtainable image quality and resolution. In 1931, Maria Goeppert-Mayer predicted molecular two-photon excitation and paved the way for multiphoton-based deep tissue intravital imaging, established in 1990 [83], [84]. The basic principle of multiphoton microscopy is the excitation of a fluorophore with simultaneous absorption of two or more photons. It belongs to the subset of non-linear microscopy, as the amount of absorbed light is proportional to the square of excitation intensity [85]. Due to the weak nature of this photon interaction, multiphoton microscopy depends on the use of femtosecond pulsed lasers, creating high instantaneous intensities [86].

In conventional laser scanning microscopy, fluorophores are excited by a laser source at their designated wavelength. Excitation leads to an elevation from their energetic ground state to an excited state. The fluorophore then emits fluorescence as it returns to the ground state, which is detected by the microscope (Fig.5A). For conventional techniques like widefield or confocal microscopy, fluorescence occurs in the focal plane but also in the background.

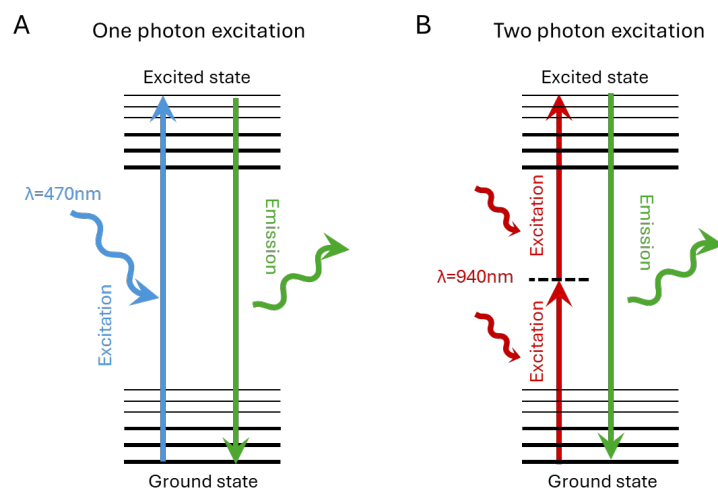


Figure 5: Jablonski diagrams for conventional one-photon and two-photon excitation. (A) For conventional excitation of fluorophores, a single photon elevates the molecule into the excited state. Fluorescence is released and the molecule relaxes back to the ground state. (B) For two-photon excitation, two photons of approximately half the energy arrive simultaneously and elevate the fluorophore to an excited state via a virtual state (dashed line). The emitted fluorescence is identical to that in conventional excitation.

For multiphoton microscopy, two or more photons of lower energy are used for excitation. A pulsed laser source enables multiple photons to arrive simultaneously and provide sufficient energy to elevate the fluorophore into an excited state. The emitted fluorescence does not change properties and remains at the same energy level as for conventional microscopy (Fig. 5B). Due to the difference in excitation, multiphoton microscopy requires a different laser source. A pulsed infrared laser, usually a titanium-sapphire laser, provides excitation wavelengths ranging from 700-1300 nm. The light at longer wavelengths contains less energy and is especially beneficial for intravital imaging as it decreases phototoxicity. Longer wavelengths also cause less light scattering and therefore enable deeper tissue penetration. The excitation of fluorophores only occurs in the focal plane, making the process confocal by default. This allows detection of all emitted light as it originates from the focal plane only and therefore improves signal efficiency.

With the commercial availability of multiphoton microscopy platforms in the early 2000s, research groups started using them for intravital imaging due to their superior imaging modalities for living specimens. Pioneer work for intravital kidney multiphoton microscopy focused on the juxta-glomerular apparatus, portraying the effects of tubular flow on the glomerular filtration rate [87]. Intravital imaging enables studying the complexity of the glomerular structures, including multi-cell composition, perfusion and shear stress due to filtration in their designated environment. With multiphoton microscopy, it is possible to obtain data on dynamic intracellular processes, like podocyte calcium flux, but also follow physiological processes over time, for example, cell lineage tracing with serial imaging [23], [88], [89].

1.4.2 Super-resolution stimulated emission depletion (STED) microscopy

In 1834, Ernst Abbe postulated a theoretical diffraction limit for optical resolution. The resolvable distance d between two points, λ as the wavelength of emitted light and NA as the numerical aperture (NA) of the objective, are related through the following equation:

$$d = \frac{\lambda}{2 * NA}$$

In theory, this limits the resolution for fluorescent microscopy to roughly 200 nm. To further increase resolution, the two options are either increasing the numerical aperture of the objective or using light with a shorter wavelength. The NA of modern high-NA oil immersion objectives reaches a maximum of 1.44. The low wavelength, high-energy electron beams used for electron microscopy are not live-cell compatible and therefore limits its application for any molecular imaging of dynamic processes. In 2014, the Nobel Prize was awarded for the development of super-resolution imaging techniques, advancing the possibilities of microscopy for life science applications to a higher resolution below the diffraction limit. One of those techniques is stimulated emission depletion (STED) microscopy [90]. STED microscopy uses a conventional confocal-like microscope setup with a focused excitation light source, a pinhole, and laser-scanning across the sample, but with an additional STED laser beam [91].

STED microscopy uses a simple principle to obtain resolution below the diffraction limit: When exciting fluorophores, the scale of the excitation beam is around 200 nm, and all fluorophores in this range emit fluorescence simultaneously. To distinguish features below that range, some fluorophores need to be kept in their dark, non-fluorescing off state. To achieve this, a donut-shaped depletion laser suppresses fluorescence via stimulated emission depletion (Fig. 6B). Photons with energy matching the gap between excited and ground state stimulate the fluorophore to release photons. The characteristics of those stimulated photons are identical to the photon of the STED beam that triggered the emission, enabling a spectral differentiation from spontaneous fluorescence emission [91].

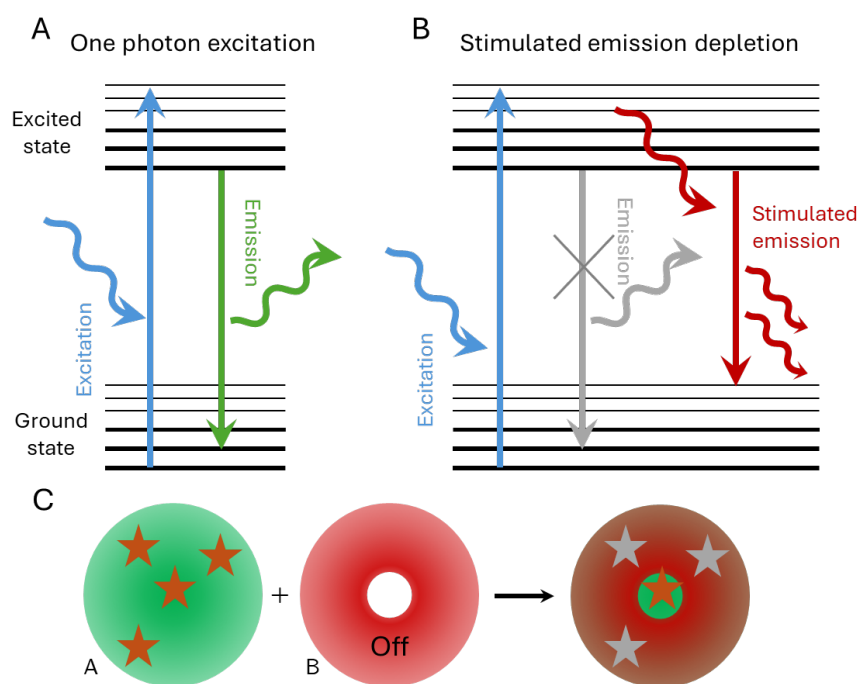


Figure 6: Stimulated emission depletion principle for fluorescence microscopy. (A) During conventional excitation, photons elevate the fluorophore into an excited state. Fluorescence is emitted and the molecule relaxes back to ground state. (B) Jablonski diagram for stimulated emission depletion after excitation. Instead of emitting fluorescence, the molecule is stimulated again with a photon of lesser energy and they are silenced by stimulated emission. No emission of fluorescence can take place. (C) Laser beam set up for STED microscopy. Single fluorophores (stars) are in their on-state (orange) by exciting them with laser setup (A). The STED laser beam (B) is donut-shaped and turns fluorophores into their off state by stimulated emission depletion. The resulting combination enables only fluorophores around low STED intensity to emit fluorescence, thus creating a region with sub-diffraction limit resolution. Modified after [92]

After initial excitation, the additional STED beam allows only a subset of molecules in its centre to emit fluorescence due to its donut shape. It thereby achieves sub-diffraction resolution by silencing the rest of the fluorophores by stimulated emission depletion.

Further improvements for resolution can be made by optical clearing of tissue due to reduced light scattering and refractive index matching [93]. Other improvement options are deep and machine-learning algorithms used for the deconvolution of obtained STED images. One disadvantage of STED microscopy is the need for high STED beam laser power, leading to high phototoxicity, making it only partially live cell compatible. Advancements in imaging modalities, however, have already improved the application range of STED imaging for 3D-tissue imaging and increased depth penetration [94], [95], [96], [97].

Resources

Images were processed using FIJI and Omero.figure web application. Figures were assembled and created using Adobe Illustrator (Version 29.7.1) or Microsoft® PowerPoint® (Version 2408 Build 16.0.17932.20742). For statistical analysis of data, the software Graph Pad Prism (Version 10.6.1) was used. Grammarly (Version 6.8.263) was used for spell & grammar checking. Mendeley Reference Manager (Version 1.19.8) was used for the management and insertion of references & bibliography. Other materials & methods used are listed in each manuscript.

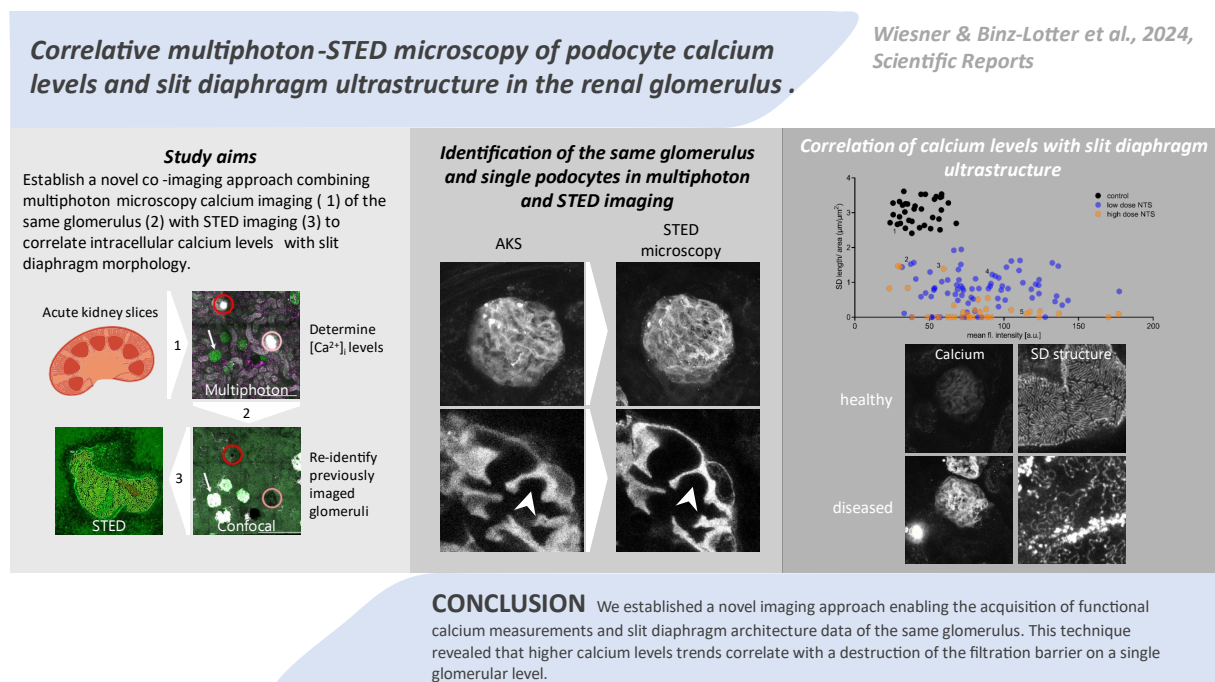
Results

This result chapter consists of two peer-reviewed publications and one unpublished manuscript. The first publication focuses on technical aspects of kidney imaging, especially multiphoton and STED microscopy and how using both can help to study the relationship between calcium levels and podocyte morphology. Both microscopy techniques were key elements of my thesis work presented here. The second publication characterizes the novel Lifeact.mScarlet-I mouse model. The data confirms correct integration of the reporter construct into the mouse genome, investigates effects of reporter expression on renal function and lastly, confirms bright, specific fluorescent labelling of actin structures. The third, unpublished manuscript utilizes this new reporter to investigate actin in diseased podocytes to study FPs effacement and its effect on the slit diaphragm.

2.1 Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus.

Wiesner Eva, Binz-Lotter Julia, Hackl Agnes, Unnersjö-Jess David, Rutkowski Nelli, Benzing Thomas, Hackl Matthias J. *Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus*. Sci Rep. 2024 Jun 6;14(1):13019. doi: 10.1038/s41598-024-63507-9. PMID: 38844492; PMCID: PMC11156906.

Graphical Abstract



Previous work showed a connection between glomerular calcium levels and FP effacement. However, most data was collected in isolated glomeruli models or obtained from cell culture experiments, lacking the intricate setup of podocytes in the environment of the glomerular apparatus. Therefore, we were interested in establishing a method to visualize both calcium dynamics and ultrastructural features in living tissue, *ex vivo*. In acute kidney slices (AKS), the podocyte architecture remains undisturbed and the multicellular environment is close to physiological conditions. We utilized multiphoton microscopy to address the question of how intracellular calcium levels are altered in AKS during NTS nephritis. Since the resolution of multiphoton microscopy regarding the slit diaphragm was limited, we established a novel

workflow that enabled us to image the same glomerulus with successive super-resolution STED microscopy. Consecutive imaging of intracellular calcium levels with multiphoton microscopy and respective foot process effacement with STED microscopy in the same glomerulus allowed us to study the relationship of both in a disease model.

Author contributions

- E.W. performed all experiments, collected and analyzed the data, wrote the manuscript and generated the manuscript figures. The technique was established during the execution of E.W.'s master thesis in the Nephrolab. Parts of the data for control and high dose NTS in Fig. 3B+D and the panels of Fig. 1B were included in the master thesis (for B: n=1 out of 4 mice for Control and n=2 out of 5 mice for high dose NTS; for D: n=2 out of 4 mice for high dose NTS).
- J.B.-L. designed experiments, revised the manuscript, provided guidance and supervision.
- A.H. inspired the project concept and revised the manuscript.
- D.U.-J. provided guidance and teaching for STED microscopy.
- N.R. revised the manuscript.
- T.B. provided funding and guidance.
- M.J.H. provided supervision, funding and revised the manuscript.



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Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus

Eva Wiesner^{1,2,4}, Julia Binz-Lotter^{1,2,4}, Agnes Hackl^{1,3}, David Unnersjö-Jess^{1,2}, Nelli Rutkowski^{1,2}, Thomas Benzing^{1,2} & Matthias J. Hackl^{1,2}✉

In recent years functional multiphoton (MP) imaging of vital mouse tissues and stimulation emission depletion (STED) imaging of optically cleared tissues allowed new insights into kidney biology. Here, we present a novel workflow where MP imaging of calcium signals can be combined with super-resolved STED imaging for morphological analysis of the slit diaphragm (SD) within the same glomerulus. Mice expressing the calcium indicator GCaMP3 in podocytes served as healthy controls or were challenged with two different doses of nephrotoxic serum (NTS). NTS induced glomerular damage in a dose dependent manner measured by shortening of SD length. In acute kidney slices (AKS) intracellular calcium levels increased upon disease but showed a high variation between glomeruli. We could not find a clear correlation between intracellular calcium levels and SD length in the same glomerulus. Remarkably, analysis of the SD morphology of glomeruli selected during MP calcium imaging revealed a higher percentage of completely disrupted SD architecture than estimated by STED imaging alone. Our novel co-imaging protocol is applicable to a broad range of research questions. It can be used with different tissues and is compatible with diverse reporters and target proteins.

Within the kidney, glomeruli are the functional filtration units consisting of different cell types such as mesangial cells, fenestrated endothelial cells, and podocytes. Podocytes are terminally differentiated epithelial cells lining the urinary site of the filtration barrier in the glomerulus. Podocytes consist of a large cell body with primary processes, from which smaller foot processes extend and enwrap the entire surface of glomerular capillaries. Neighboring foot processes interdigitate and are connected by a specialized cell-cell junction termed slit diaphragm (SD), containing unique proteins like nephrin and podocin^{1,2}. The SD functions as a signaling hub and controls the podocyte's complex cytoskeletal architecture³. Podocyte injury results in flattening and broadening of foot processes, called effacement, and podocyte detachment from the glomerular basement membrane. This is a major contributor to kidney disease development, progression and failure, as lost podocytes cannot be replaced, resulting in the clinical disease phenotype of focal segmental glomerulosclerosis. Human mutations in SD proteins, the actin-binding protein α -actinin-4, and the transient receptor cation channel 6 (TRPC6), regulating calcium signaling, result in the same phenotype of focal segmental glomerulosclerosis^{4–6}. Besides TRP channels, podocytes are capable of purinergic calcium signaling whose activation results in increased intracellular calcium levels, underlining the importance of calcium, actin and the SD interplay for podocyte biology^{7–10}. We have previously shown, using in vivo MP calcium imaging, that calcium levels in podocytes increase upon injury¹¹. Similar results applying MP microscopy have been obtained ex vivo, using acute kidney slices (AKS), which provide a multicellular environment of vital intact kidney tissue¹². The use of isolated glomeruli is limited

¹Department II of Internal Medicine and Center for Molecular Medicine Cologne, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany. ²Cluster of Excellence Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany. ³Department of Pediatrics, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany. ⁴These authors contributed equally: Eva Wiesner and Julia Binz-Lotter. ✉email: Matthias.hackl@uk-koeln.de

for studying podocyte calcium levels as glomerular isolation induces high baseline calcium levels as a sign of podocyte injury¹¹.

MP imaging uses a pulsed laser to excite fluorophores with multiple photons of a long wavelength. This allows for deeper tissue penetration especially as kidney tissue is highly scattering. However, as a light microscopy technique its resolution is limited by the diffraction limit of light. The gold standard for imaging of the SD has been electron microscopy, as the 30–40 nm width of the SD cannot be resolved by a conventional confocal microscope¹³. Super-resolution techniques like STED microscopy allow visualization and quantification of SD proteins after immunofluorescent labeling (Suppl. Fig S1)^{14–16}. During image acquisition an additional donut-shaped stimulated emission depletion laser minimizes the area of fluorescence and thereby increases resolution¹⁷.

So far, functional calcium and structural SD data are obtained in separate experiments, prohibiting correlative studies in single glomeruli. In vivo STED imaging and MP coupled with STED (MP-STED) on a single microscope have been demonstrated but are not commercially available^{18–21}. Additionally, in vivo STED imaging requires expression of fluorescent reporters and lacks the possibility of immunofluorescent staining. MP-STED imaging uses mostly fixed tissues, thus does not allow for imaging of live calcium dynamics.

To investigate how podocyte calcium levels and SD architecture are interlinked within one glomerulus in the transition from health to disease states, we established a new correlative imaging approach. It combines vital tissue and high-resolution imaging using MP and STED microscopy sequentially without the need for a combined MP-STED system. We applied our approach to compare podocyte calcium levels and SD architecture in control mice and mice treated with NTS. Injection of NTS leads to an autoimmune response against glomerular structures, resulting in podocyte injury.

Results

To combine MP and STED microscopy to measure podocyte calcium levels and SD morphology within the same glomerulus, we prepared AKS from mice expressing the calcium indicator GCaMP3 exclusively in podocytes^{22,23}. The experimental imaging setup of our novel protocol is outlined in Fig. 1. A small mark in the AKS enabled identical slice orientation in both imaging modalities. During MP imaging, burn marks were positioned in safe proximity to glomeruli as reference points and large three-dimensional tile scans were acquired and served as an overview. In a single field of view z-stacks of single glomeruli were obtained to measure the mean GCaMP3 fluorescence, as a readout for calcium levels in podocytes. After MP imaging, AKS were fixed, the tissue was cleared and stained with antibodies against the SD protein nephrin and the green fluorescent protein (GFP), as the required clearing step resulted in complete loss of GCaMP3 fluorescence. The added reference points allowed the mapping of each glomerulus in the two data sets. Then, confocal image stacks were acquired to confirm morphology of previously imaged glomeruli. In the following image acquisition step, super resolution images of the nephrin pattern of those glomeruli were obtained with the activated STED laser. Finally, we quantified nephrin staining patterns as recently published by Butt et al. by measuring the SD length covering a capillary surface area¹⁵. This results in a numerical value for SD length/area, which has been shown to decrease in disease states in mice and humans compared to healthy controls.

Comparison of image stacks acquired by MP microscopy and stacks acquired with confocal settings on a STED microscope confirmed the overall conservation of tissue morphology (Fig. 2a MIP, Suppl. Video 1). We were able to match, both podocyte cell bodies (Fig. 2a, asterisks) and primary processes (Fig. 2a, arrows) in single planes in control animals in both imaging modalities and thus validated the possibility of studying the same glomerulus with both imaging modalities. We then applied our protocol to mice injected with a low dose (8 µl/g) or a high dose (11 µl/g) of NTS. Comparable to control tissue, we were able to identify corresponding podocyte cell bodies and processes in disease (Fig. 2b, shown for high dose NTS, 11 µl/g).

Using our protocol, we obtained data on podocyte calcium levels and structural data of the slit diaphragm architecture in single glomeruli. Analysis of the calcium data revealed that animals of both NTS groups showed significantly elevated calcium levels compared to controls. However, there was no significant difference between the low and high dose NTS groups (Fig. 3a, correlative MP + STED). Analysis of the STED data of sequentially imaged glomeruli showed a significant reduction of slit length in both disease groups (high and low dose NTS) compared to control. Additionally, the higher NTS dose (11 µl/g) decreased the SD length further, indicating dose-dependent damage to the podocyte architecture (Fig. 3c). Importantly automatic and manual analysis of SD length failed in both NTS groups in a subset of glomeruli due to the complete loss of a visible SD structure caused by extensive damage (Fig. 3c, correlative MP + STED). This was the case in 13% of measurements of mice treated with low dose NTS and in 42% of measurements with high dose NTS. For analysis of these glomeruli, the SD length was set to zero.

We then compared these results to previously performed experiments, where we independently performed either only calcium imaging or only STED imaging in mice treated with high dose NTS (11 µl/g). As expected, intracellular calcium levels were equally low in control animals and increased comparably after the administration of NTS in both experiments (Fig. 3a, b). However, a comparison of the SD architecture acquired during correlative imaging and STED imaging alone revealed major differences (Fig. 3c, d). In control animals, the measured SD length/area did not differ significantly between both experiments. However, in animals treated with high dose NTS (11 µl/g) the mean SD length/area was significantly reduced in the glomeruli imaged with our correlative imaging approach compared to STED imaging alone (Fig. 3c, d). This was mainly due to the high percentage of glomeruli with complete loss of SD structure.

Finally, we investigated a relationship between intracellular calcium levels and SD structure. Therefore, we plotted intracellular calcium levels against SD length/area for each glomerulus measured in our correlative imaging approach. The data did not show a clear correlation (Fig. 4a). In more detail, the plot shows, that 71.1% of glomeruli after low dose NTS and 63.9% of glomeruli after high dose NTS had calcium levels exceeding the

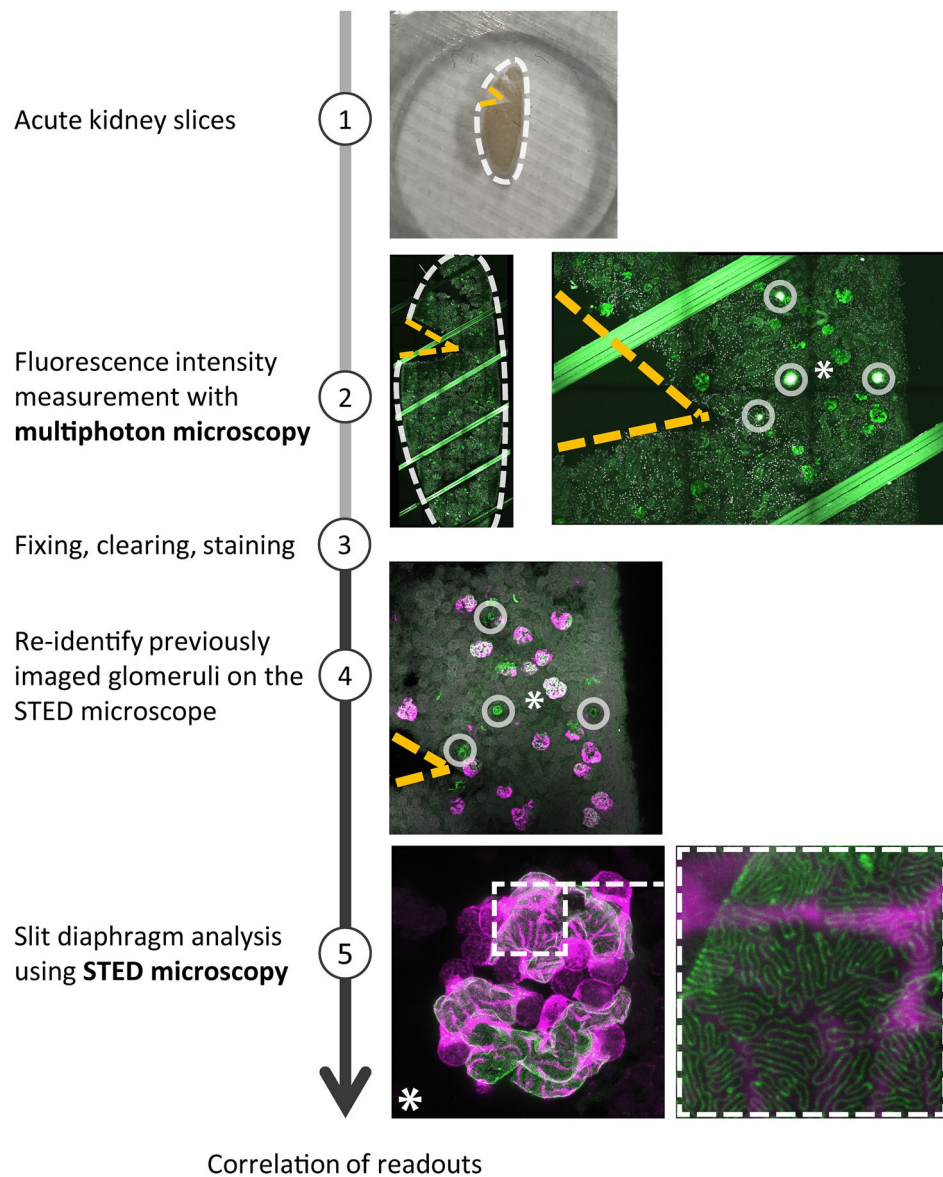


Figure 1. Workflow for correlative imaging of murine glomeruli using MP and STED microscopy. Overview of sample preparation and imaging steps. (1) AKS are marked by cutting a triangular shape at the upper left corner. (2) Intensity measurements with MP microscopy with burn marks as reference points (circles) and surrounding glomeruli (asterisk). The green channel shows endogenous GCaMP3 fluorescence and propidium iodide staining in white. (3) Fixation, clearing and staining of slices with goat anti-nephrin primary antibody, donkey anti-goat Alexa-594 secondary antibody and anti-GFP-Alexa647. (4) Confocal images with reference points (circles) and previously imaged glomeruli (asterisks). (5) STED images of the glomerulus marked with asterisk. Anti-GFP staining in magenta labelling GCaMP3 and anti-nephrin staining in green.

maximum calcium levels of controls (indicated by the dashed line in Fig. 4a). While SD damage increased gradually with higher NTS doses, intracellular calcium levels did not. Calcium levels displayed a large variance and showed no significant correlation with the extent of SD damage. Representative images of SD morphology and GCaMP3 fluorescence for selected glomeruli of the different groups are shown in Fig. 4b.

Discussion

Our novel protocol allows visualization and correlation of functional and ultrastructural information on the level of individual glomeruli. Adding reference points to the AKS tissue by photo ablation during MP imaging enabled the identification of the same glomerulus in STED imaging. It avoided the bias of selecting only glomeruli with residual identifiable SD structure during STED imaging and demonstrated no correlation between intracellular podocyte calcium levels and slit diaphragm architecture. Our data shows, that all glomeruli of control animals exhibited low intracellular calcium levels as previously shown *in vivo* without spontaneous calcium fluctuations as

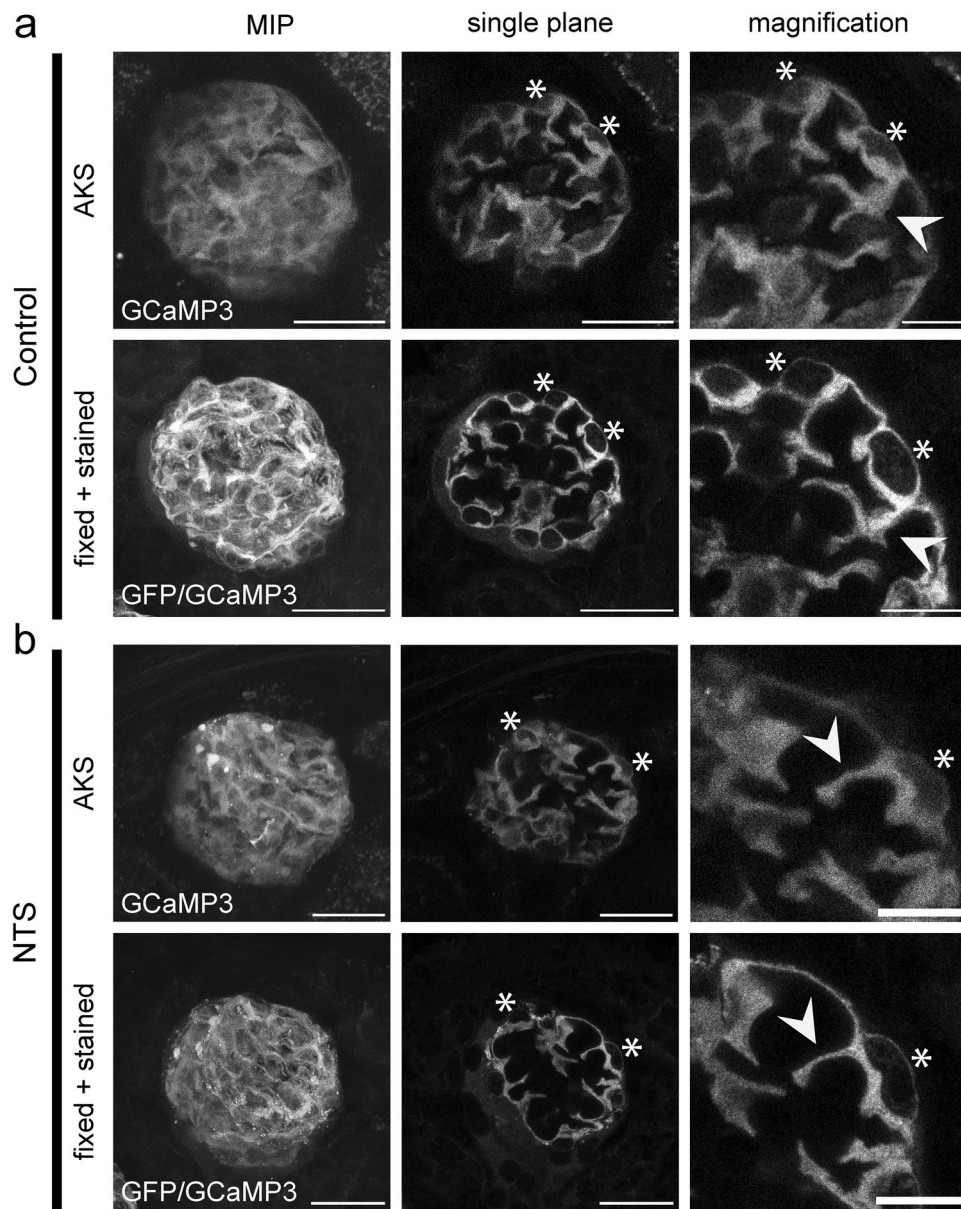


Figure 2. Reidentification of single mouse glomeruli and individual podocytes in AKS and after fixation, clearing and staining. Images of a control animal (a) and a NTS-treated mouse (11 μ l/g body weight) (b) Images show the same glomerulus – from left to right – maximum intensity projection (MIP) of the whole glomerulus (left), single plane view (middle) and magnification of single podocytes (right). Endogenous GCaMP3 fluorescence for MP images (AKS) and anti-GFP/GCaMP3 signal for confocal images (fixed + stained). Arrowheads indicate podocyte primary processes. Asterisk indicating single podocyte cell bodies. Scale bar MIP & single plane – 25 μ m; magnification – 10 μ m.

described in tubular cells^{11,24}. To exclude effects on intracellular calcium levels due to the experimental approach we incubated AKS with propidium iodide to label dead cells prior to calcium imaging. Thereby we excluded high calcium levels due to tissue damaged caused by the cutting process or tissue degeneration. Additionally, staining with propidium iodide did not interfere with STED imaging. After induction of NTS nephritis, low and high dose NTS groups showed a similar increase in calcium levels as measured by mean GCaMP3 fluorescence, but with a large variation between glomeruli. In a second set of experiments (Fig. 3c, only MP) the mean fluorescence and the variation of calcium levels were comparable in the control and high dose NTS groups. In addition, NTS had a pronounced dose-dependent effect on SD architecture. Therefore, we are convinced that the NTS model yields reproducible results and the variation in calcium levels is not due to a sampling error of differently affected glomeruli. Nevertheless, we did not find a correlation between increased intracellular calcium levels and the destruction of SD architecture in disease (Fig. 4a). While intracellular calcium levels can change dynamically, morphological changes to the SD take far longer to form and resolve²⁵. Therefore, calcium measurements at a

single time point could be insufficient to determine the extent of glomerular damage, even if the tight regulation of podocyte calcium levels is lost in disease and mean calcium levels increase. Additionally, the application of a membrane-bound calcium sensor might provide further insights into the calcium dynamics in closer proximity to the SD and thus result in a stronger correlation.

We previously demonstrated that injured podocytes are sensitized to calcium signaling upon stimulation¹¹. Therefore it is an intriguing possibility, that not the basal calcium levels, but the susceptibility to calcium signaling itself has a close relationship with the degree of damage. Adding a perfusion pump to exchange buffers to our setup would allow for triggering dynamic calcium responses with compounds such as angiotensin II or ATP and correlate them to podocyte ultrastructure by STED microscopy to test this hypothesis.

Additionally, our correlative imaging approach revealed that in nearly half of the glomeruli in the high dose NTS group nephrin abundance was diminished to such a degree that analysis of SD length by STED imaging was not possible (Fig. 3c). The main difference between correlative imaging and STED imaging alone is the selection of glomeruli during initial calcium imaging. The fact that the mean fluorescence of the calcium indicator GCaMP3 was comparable with a previous set of experiments argues against a selection bias towards highly damaged glomeruli in this step. Glomeruli with a complete disruption of the SD architecture lack the continuous nephrin staining pattern and they are likely to be overlooked during STED imaging alone. By picking intact glomeruli in living tissue during the first imaging step, we eliminated the selection bias based on the recognizability of the slit diaphragm pattern. Thus, we acquired glomeruli with more extensive SD damage than STED microscopy alone would (Fig. 3c, d). Therefore, the published SD analysis protocol presumably underestimates the severity of alterations in podocyte architecture in advanced cases of glomerular disease¹⁵.

Other groups also reported the use of MP microscopy systems with an implemented STED laser beam for increased resolution, but as no fluorescent reporter of the SD is available, this does not allow visualization of the SD^{19,20,26}. In contrast our correlative imaging approach combines vital tissue calcium imaging and high-resolution imaging of antibody labelled proteins within the same sample. Although it requires two imaging sessions, separate MP and STED microscopes are more widespread available than the combination of both lasers in a single microscope.

The increasing availability of genetically encoded fluorescent sensor proteins for in vivo imaging and immunofluorescent labeling options for STED microscopy make our correlative workflow suitable for answering a broad range of research questions far beyond the glomerulus in the kidney. Additionally, by using mosaic expression to obtain only a few positive cells of interest, the resolution could be further improved to correlate microscopy results on the level of individual cells²⁷.

In conclusion, we present a protocol for correlative imaging of AKS with MP and STED microscopy to correlate functional and structural information on the level of individual glomeruli.

Methods

Mice

Pod:cre-GCaMP3 (C57Bl6/N) mice of both sexes at the age of 4 weeks were used. Control mice (n = 3) received no treatment; for NTS nephritis induction animals received a single i.p. injection of NTS (Probetex, PTX-001-S, San Antonio, TX, USA): low dose 8 µl/g (n = 4) or high dose 11 µl/g (n = 3). No method of randomization or blinding was used. Mice were bred and kept in individually ventilated cages under specific-pathogen free conditions in an in vivo research facility (CECAD, Cologne). Mice were monitored for weight loss and proteinuria starting on day 3 (not shown). After 5 days mice were euthanized by cervical dislocation and the kidneys were harvested. All experiments were approved by the LANUV NRW, Germany, with the authorization number: VSG 81-02.04.2018.A351. Animal experiments complied with LANUV regulations and the ARRIVE guidelines²⁸.

Preparation of acute kidney slices (AKS) and MP microscopy

Freshly harvested mouse kidneys were embedded in low-melting agarose (4%) and cut into 300 µm slices using a vibratome (Leica, VT1200 S). Slices were maintained in Krebs-Henseleit-Buffer carbogenated with 95% O₂/5% CO₂ before and during the experiment²⁹. To enable identical slice orientation in both imaging modalities, a triangle-shaped piece of tissue was cut from the AKS at the upper left corner. To ensure tissue integrity and exclude glomerular damage caused by cutting, AKS were incubated with propidium iodide (5 µM, Sigma) for 5 min. Glomeruli, which contained cells with nuclear propidium iodide staining, were excluded from the analysis. MP imaging was performed with a TCS SP8 MP-OPO microscope (Leica Microsystems) with a Chameleon Vision II laser (Coherent) tuned to 940 nm within a maximum of 5 h after harvesting the kidneys. Burning marks for reorientation were created by laser ablation using the 'bleach point' tool of the Leica Software. Single field of view z-stacks of glomeruli were acquired with 512 × 512 pixel and around 50–60 µm total stack size. After MP imaging, all AKS were fixed with 4% formaldehyde overnight.

Preparation of acute kidney slices for STED microscopy

Immunofluorescent labeling was conducted as previously described¹⁴. Shortly, all fixed AKS were optically cleared, followed by consecutive primary and secondary antibody incubation in PBS-T (0.1% Triton-X100 in PBS) for 24 h at 37 °C. If only STED microscopy was performed a piece of the kidney was immersed in hydrogel, the gel was polymerized for 3 h at 37 °C and ultimately washed with PBS. The piece was then cut into 200 µm slices using a vibratome (Leica, VT1200 S). The slices were then processed in an identical way as AKS.

Primary antibodies used: guinea pig anti-nephrin (Fitzgerald, 20R-NP002, 1:100), anti-GFP-Alexa647 (Thermo Fisher, A-31852, 1:100) for labelling GCaMP3. Secondary antibody: donkey anti-guinea pig Atto594 (coupled in house, 1:100). Confocal and STED images of fixed samples were acquired with a TCS SP8 gSTED 3X microscope (Leica Microsystems) equipped with a white light laser and a 775 nm 2D donut STED depletion

Figure 3. Correlative imaging reveals a stronger reduction of SD length/area than STED imaging alone. **(a)** Quantification of mean fluorescence (fl.) intensities of glomeruli for control and NTS-treated mice using our correlative MP+STED workflow. Control (n = 35 glomeruli of 3 mice), low dose NTS (8 µl/g, n = 76 glomeruli of 4 mice) and high dose NTS (11 µl/g, n = 36 glomeruli of 3 mice). **(b)** Quantification of mean fluorescence intensities of glomeruli for control and NTS-treated mice using only MP microscopy. Control (n = 228 glomeruli of 4 mice) and high dose NTS (11 µl/g, n = 578 of 5 mice). **(c)** Quantification of SD length/area of healthy control, low dose NTS (8 µl/g, n = 76 glomeruli of 4 mice) and high dose NTS (11 µl/g, n = 36 glomeruli of 3 mice). For each glomerulus, one STED image was acquired. Glomeruli in which no SD length could be measured were set to value = 0. **(d)** Quantification of SD length/area of healthy control and NTS-treated mice using STED microscopy only. Control (n = 37 glomeruli of 4 mice) and high dose NTS (11 µl/g, n = 29 glomeruli of 4 mice). Statistical analysis: ordinary one-way ANOVA with Tukey's multiple comparisons test; p-values: <0.1234 (ns), <0.0332 (*), <0.0021 (**), <0.0002 (***), <0.0001 (****). The red line represents mean ± SD. AKS acute kidney slices, NTS nephrotoxic serum, SD slit diaphragm, ns not significant.

laser (Leica Microsystems). The excitation wavelengths were: $\lambda_{\text{Ex}} = 594$ nm for Atto594 and $\lambda_{\text{Ex}} = 650$ nm for Alex647, channels were acquired sequentially. We obtained images with a resolution of 0.011 µm per pixel, with a 2048 × 2028 pixel image size. For each glomerulus, only one nephrin-stained capillary surface was acquired as close to the coverslip as possible with a z-stack size of approximately 2–4 µm depending on the orientation of the capillary.

SD length analysis

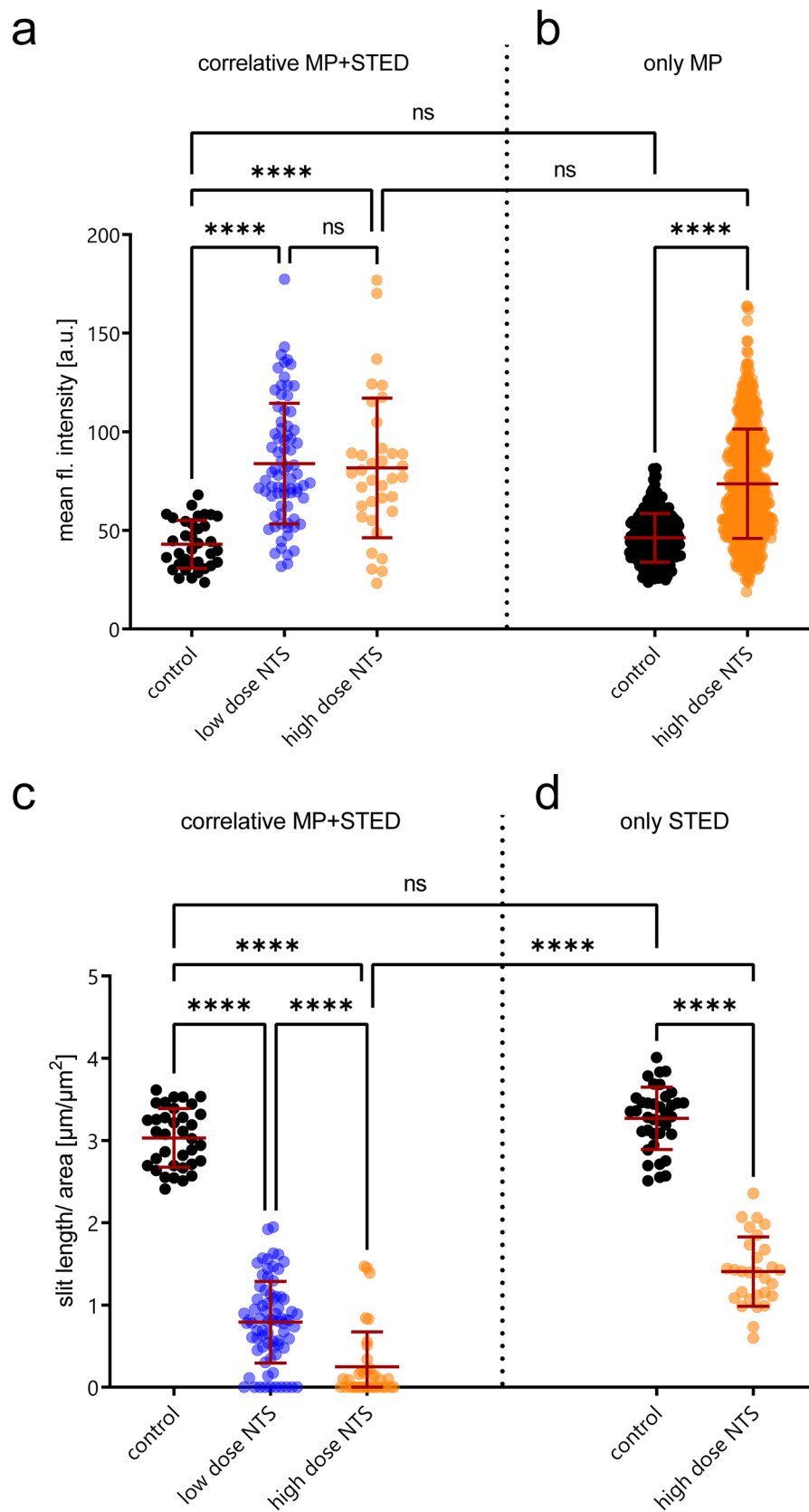
SD length was measured using a previously published macro¹⁵. The analysis was conducted as described in detail in the original publication. The macro itself is deposited on GitHub: <https://gist.github.com/github-martin/e699d18ae6cfa5b1a6aace15d3c3544c>. In a few cases, where the image contrast was insufficient to use the macro, the SD length was measured manually using FIJI.

Calcium measurements

Calcium levels in podocytes were measured using FIJI and analyzed as previously published¹¹. Briefly, maximum intensity projections of z-stacks of glomeruli were manually annotated with an ROI comprising the glomerular area. The mean fluorescence intensity of this ROI was used as a readout.

Statistics

For statistical analysis of mean fluorescence intensities and SD length per area, we used ordinary one-way ANOVA with Tukey's multiple comparisons test. No data points were excluded. P values < 0.05 were considered statistically significant.



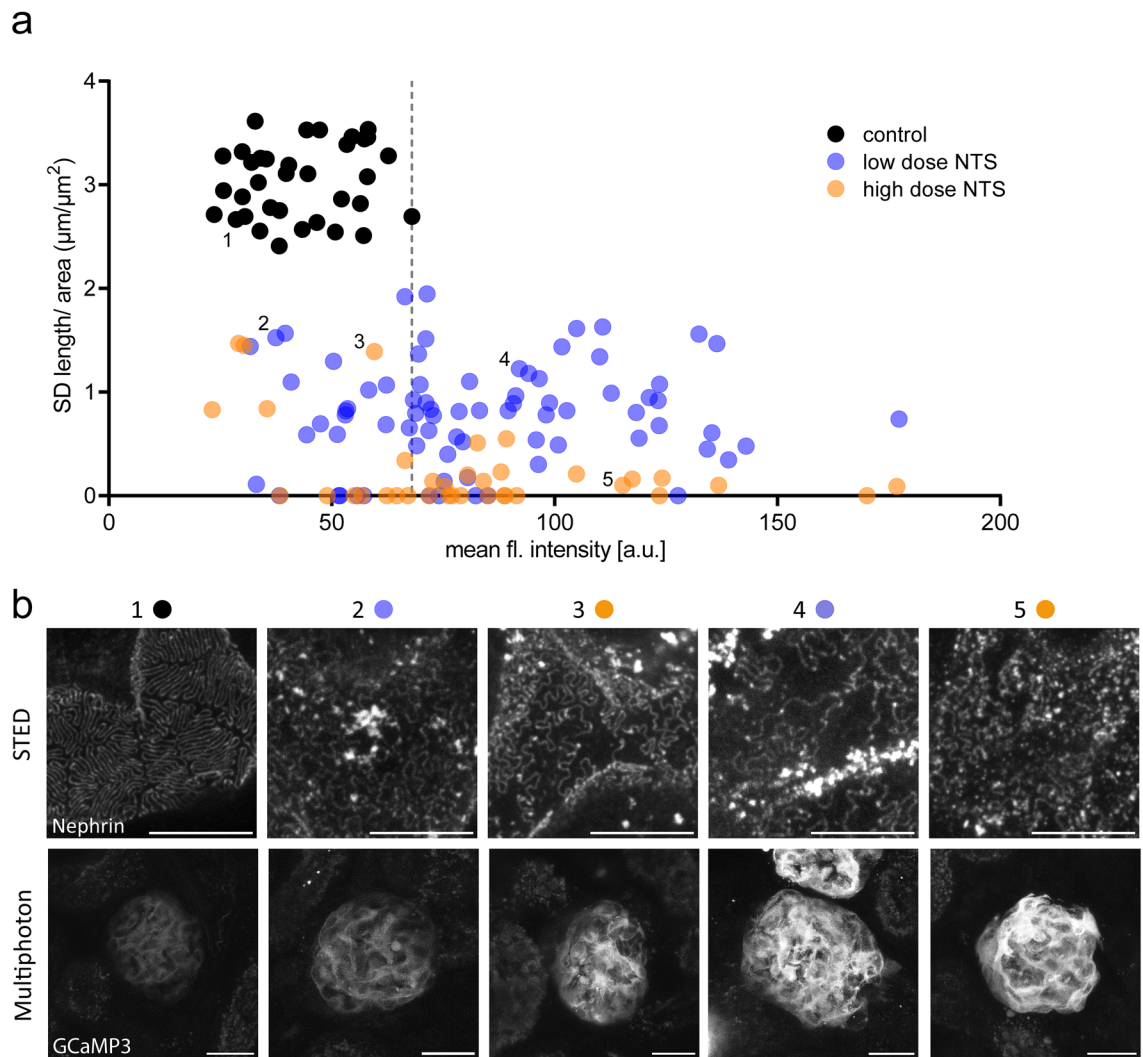


Figure 4. Correlation of intracellular calcium levels and podocyte slit diaphragm ultrastructure. **(a)** Correlation of mean GCaMP3 fluorescence intensities and slit diaphragm length/area for individual glomeruli. The dashed line indicates the maximum GCaMP3 fluorescence intensity measured in control animals. Control: $n = 35$ glomeruli of 3 mice; low dose NTS: $n = 76$ glomeruli of 4 mice; high dose NTS: $n = 36$ glomeruli of 3 mice. **(b)** Representative MIP images of slit architecture acquired using STED imaging (upper panels) and GCaMP3 fluorescence in MP microscopy (lower panels) of the same glomerulus. Symbols above panels show respective positions in panel (a). Scale bar STED – 5 μm ; MP images – 25 μm ; MIP – maximum intensity projection.

Data availability

The datasets generated and analyzed during the current study are available in the figshare repository, <https://figshare.com/s/79be57e74e3fe693876b> with the reserved <https://doi.org/10.6084/m9.figshare.24118053>.

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Author contributions

Calcium MP and STED imaging experiments were designed by J.B.L., A.H. and M.J.H. Experiments were performed by E.W., N.R. and J.B.L. Data analysis was carried out by E.W. and D.U.-J. The manuscript was written by E.W., J.B.L. and M.J.H. T.B. and M.J.H. provided guidance and resources.

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Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to M.J.H.

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Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus.

Eva Wiesner^{1,2,4}, M.Sc., Julia Binz-Lotter^{1,2,4}, PhD, Agnes Hackl^{1,3}, MD, PhD, David Unnersjö-Jess^{1,2}, PhD, Nelli Rutkowski^{1,2}, M.Sc., Thomas Benzing^{1,2}, MD, *Matthias J. Hackl^{1,2}, MD.

¹Department II of Internal Medicine and Center for Molecular Medicine Cologne, University of Cologne, Faculty of Medicine University Hospital Cologne, Germany.

²University of Cologne, Faculty of Medicine and University Hospital Cologne, Cluster of Excellence Cellular Stress Responses in Aging-associated Diseases (CECAD), Germany

³Department of Pediatrics, University of Cologne, Faculty of Medicine and University Hospital Cologne, Cologne, Germany

⁴contributed equally

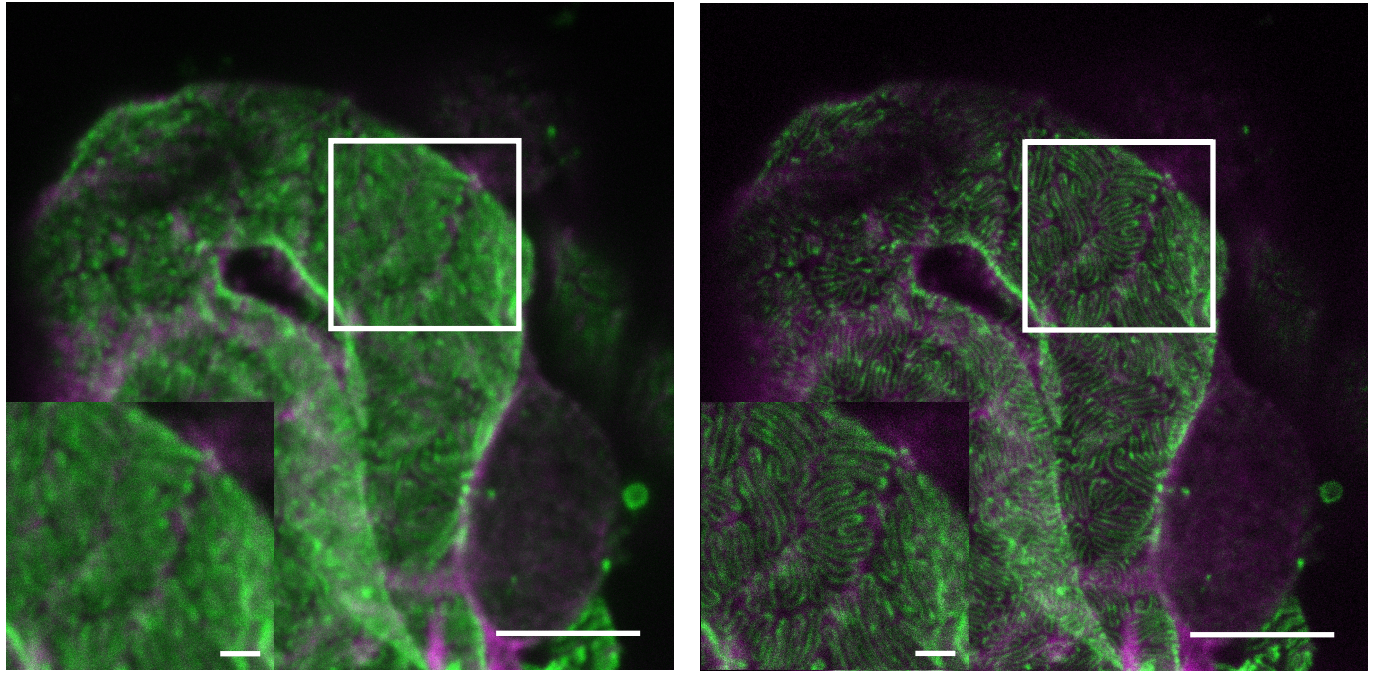
Corresponding author: Matthias J. Hackl, Matthias.hackl@uk-koeln.de, phone: +4922147832319, fax: +492214781422944

Supplementary Information

Supplemental Video 1: Structural overview of the same glomerulus imaged with a multiphoton microscope (left) and confocal settings on a STED microscope after processing (right). The left stack shows a glomerulus of a healthy mouse expressing GCaMP3 in podocytes acquired by multiphoton imaging. The right stack shows the same glomerulus after processing (fixation and staining) acquired with confocal imaging settings using a STED microscope. A comparison of both image stacks confirms the identification of the same glomerulus in both imaging modalities.

confocal

STED



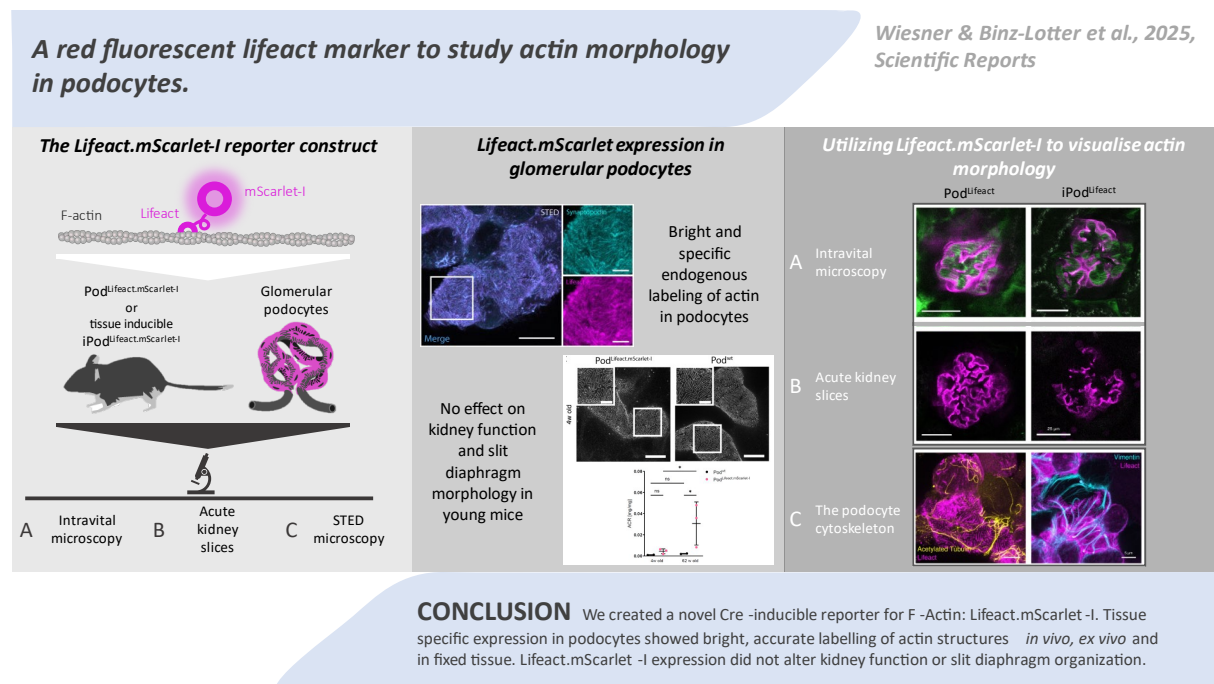
Supplemental Figure 1: Comparison of confocal and STED images of the nephrin-stained slit diaphragm. Left image shows a MIP acquired with confocal settings. Right panel shows the same area imaged with the same settings and additional STED laser beam at 775nm. Green channel shows anti-nephrin Atto594 staining. Magenta channel shows anti-GFP Alexa647 staining. Bottom left corner of images shows zoomed in area (white rectangle).

MIP – maximum intensity projection, scale bars – 5 μ m; zoom in – 1 μ m.

2.2 A red fluorescent lifeact marker to study actin morphology in podocytes.

Wiesner Eva, Binz-Lotter Julia, Tröder Simon E., Unnersjö-Jess David, Rutkowski Nelli, Zevnik Branko, Schermer Bernhard, Benzing Thomas, Wedlich-Söldner Roland, Hackl Matthias J. *A red fluorescent lifeact marker to study actin morphology in podocytes*. Sci Rep. 2025 Apr 11;15(1):12386. doi: 10.1038/s41598-025-96822-w. PMID: 40216917; PMCID: PMC11992033.

Graphical Abstract



Previous work by the Wedlich-Söldner group demonstrated that a calcium-mediated actin reset (CaAR) occurs in cultured podocytes, i.e., that the actin cytoskeleton is reorganized following a calcium increase. Our previous work showed that podocyte baseline calcium concentration increased and FPs were effaced in the NTS nephritis model. We were interested to see if calcium signals are relayed to the podocyte actin cytoskeleton by CaAR. However, it has not been possible to combine podocyte calcium and actin cytoskeleton visualization directly in living animals. Therefore, we wanted to generate a novel actin reporter mouse model suitable for labeling actin endogenously in podocytes. The small peptide Lifeact from *Saccharomyces cerevisiae* can bind actin filaments. Lifeact constructs coupled with fluorescent reporter proteins allow visualization of actin dynamics *in vivo*. Ultimately, we wanted to visualize actin simultaneously with calcium levels to investigate CaAR. Thus, we designed a novel, inducible Lifeact reporter coupled to the red fluorescent protein mScarlet-I, making it suitable for intravital imaging in combination with GCaMP calcium reporters. Our Lifeact.mScarlet-I reporter model provided new insights into healthy podocyte morphology. Additionally, this new reporter tool opens up new possibilities for future work to study the role of actin in FPs effacement. The newly generated reporter has a broad range of applications throughout life sciences and has already been transferred to groups with diverse research interests outside the field of nephrology.

Author contributions

E.W.	performed all experiments, collected and analyzed the data, wrote and revised the manuscript and prepared all manuscript figures, except Fig.1 A-C.
J.B.-L.	wrote and revised the manuscript, conducted the cloning of Lifeact.mScarlet-I plasmids for IVF, established the genotyping of Lifeact.mScarlet-I mice, performed the experiments for Fig. 1A+B, conducted the bone marrow extraction for TLA sequencing in Fig. 1C, provided supervision and guidance.
S.E.T.	conducted mouse genome editing in the ivRF of CECAD.
D. U.-J.	provided guidance for STED microscopy.
N.R.	revised the manuscript.
B.Z.	provided guidance on mouse genome editing.
B.S.	provided funding and guidance.
T.B	provided funding and guidance.
R.W.-S.	provided guidance.
M.J.H.	provided supervision, funding and revised the manuscript.



OPEN A red fluorescent lifeact marker to study actin morphology in podocytes

Eva Wiesner^{1,2,5}, Julia Binz-Lotter^{1,2,5}, Simon E. Tröder^{2,3}, David Unnersjö-Jess^{1,2}, Nelli Rutkowski^{1,2}, Branko Zevnik^{2,3}, Bernhard Schermer^{1,2}, Thomas Benzing^{1,2}, Roland Wedlich-Söldner⁴ & Matthias J. Hackl^{1,2}✉

F-actin is a major component of the cellular cytoskeleton, responsible for maintaining cell shape, enabling movement and facilitating intracellular transport. In the kidney, glomerular podocytes are highly dependent on their actin cytoskeleton shaping their unique foot processes. Hereditary mutations in actin-binding proteins cause focal segmental glomerulosclerosis, while other organs remain largely unaffected. So far, actin visualization in podocytes has been limited to electron microscopy or indirect immunofluorescent labeling of actin-binding proteins. However, the short F-actin-binding peptide Lifeact enables researchers to study actin dynamics in vitro and in vivo with minimal interference with actin metabolism. Here we introduce a new mouse model with conditional expression of a Lifeact.mScarlet-I fusion protein providing red labeling of actin. Cre recombinase-mediated activity allows cell-specific and mosaic expression in podocytes, enabling selective labeling of individual cells to contrast with non-expressing neighboring cells. Transgenic mice are born healthy and young animals display no kidney-related phenotype. By intravital imaging and super-resolution microscopy, we show subcellular localization of actin to the foot processes in a resolution previously only obtainable by electron microscopy. Our novel mouse line provides the opportunity to study the actin cytoskeleton in podocytes and other cell types by intravital imaging and other conventional light microscopy techniques.

The glomerulus forms the functional filtration unit of the kidney. Glomeruli filter plasma through a complex filtration barrier resulting in a protein-free primary urine. The filter consists of a fenestrated endothelial cell layer, the glomerular basement membrane, and a layer of epithelial cells called podocytes. Podocytes have a large cell body with protrusions termed primary processes, which further divide into smaller foot processes (FPs). FPs of neighboring podocytes interdigitate and are connected by a specialized cell-cell contact, the slit diaphragm (SD). In the SD, nephrin is one of the most prominent proteins. A sophisticated network of tubulin in the primary processes and actin bundles in the FPs support the complex podocyte structure¹.

During disease, the actin cytoskeleton drastically rearranges, leading to flattening and simplification of FPs structure, a process termed ‘foot process effacement’². Human congenital mutations in genes encoding actin-interacting proteins such as alpha-actinin-4, podocin and CD2AP result in kidney disease, while other organs show less or no phenotype, and thus highlight the significance of an intact cytoskeleton for podocyte function^{3–5}. Therefore, visualization of podocyte actin cytoskeleton has been increasingly important to understand disease development and evaluate podocyte injury².

In 2008 Riedl et. al. published Lifeact as a novel actin-binding probe. Lifeact is a 17 amino acid long peptide and binds preferentially to filamentous actin (F-actin)⁶. Since its publication, Lifeact has been fused to a variety of fluorescent proteins to label actin in vitro and in vivo. Tissue inducible expression and constitutive expression of Lifeact.EGFP and Lifeact.mRuby in mice resulted in viable, fertile and phenotypically normal animals^{7,8}.

Transient expression of Lifeact in cell culture did not alter actin dynamics⁹. Detailed structural analysis of Lifeact binding to actin using cryoEM revealed no interference with the helical symmetry of the filament¹⁰. Super-resolution imaging of Lifeact and phalloidin in HeLa cells showed comparable results for imaging quality,

¹Department II of Internal Medicine and Center for Molecular Medicine Cologne, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany. ²Cluster of Excellence Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, Cologne, Germany. ³In vivo Research Facility, Faculty of Medicine and University Hospital Cologne, University of Cologne, Cologne, Germany. ⁴Institute of Cell Dynamics and Imaging and Cells in Motion Interfaculty Centre, University of Münster, Münster, Germany. ⁵Eva Wiesner and Julia Binz-Lotter contributed equally. ✉email: Matthias.hackl@uk-koeln.de

filament thickness and length¹¹. However, Lifeact does not bind all actin forms equally, but shows a higher binding affinity towards F-actin¹². To date, only a conditional Lifeact.EGFP mouse line has been generated⁸. This limits its combination with any other green reporter protein such as GCaMP, due to overlapping emission spectra. We therefore generated a novel conditional Lifeact mouse line expressing tissue-specific Lifeact tagged with the red fluorescent protein mScarlet-I. In the group of monomeric red fluorescent proteins, mScarlet-I displays the highest calculated brightness at its fluorescence emission maximum at 569 nm, with a more than 3.5-fold increase compared to mCherry¹³. In recent years, the development of super-resolution techniques such as STED and SIM have enabled visualization of podocyte architecture similar to electron microscopy^{14,15}. Here, we demonstrate efficient endogenous actin labeling in podocytes in transgenic mice. Immunofluorescent co-staining with other proteins provides further insight into cytoskeletal structures.

Material and methods

Generation of targeting vector

For construction of the plasmid pR26 CAG LifeAct.mScarlet-I the sequence of LifeAct.mScarlet-I was cloned from the plasmid pLifeAct_mScarlet-i_N1 (Addgene #85056) in the backbone of the plasmid pR26 CAG AsiSI/MluI (Addgene #74286) using the restriction enzymes AsiSI and MluI^{13,16}. DNA Sequence of Lifeact.mScarlet-I was amplified using the forward primer 5- GCGATCGCATTCACCATGGGCGTGGCCG-3' including an AsiSI restriction site and a reverse primer 5-CGCGACGCGTCTTGACAGCTCGTCCATGCCGCC-3' including a MluI restriction site. After purification, the PCR fragment was digested and ligated into the equally digested pR26 CAG AsiSI/MluI plasmid to obtain the construct pR26 CAG LifeAct.mScarlet-I¹⁶.

Mouse genome editing

LifeAct.mScarlet-I (C57BL/6N-Gt(ROSA)26Sor^{em333}(CAG-mScarlet-I)^{Cecad}/Cecad) mice for Cre-recombinase mediated expression of LifeAct.mScarlet-I from the Gt(ROSA)26Sor locus were generated by CRISPR/Cas9 genome editing using a well-established gene targeting strategy¹⁶. Therefore, 400 nM of each Alt-R™ guide RNA and 20 ng/μl plasmid DNA repair template (pR26 CAG LifeAct.mScarlet-I) were injected with 200 nM Alt-R™ SpCas9 protein and 30 ng/μl SpCas9 mRNA (TriLink, L-6125-20) in C57BL/6Nj zygotes as previously published¹⁷. CRISPR reagents were purchased from Integrated DNA Technologies (USA) and animals were obtained from Janvier Labs (France). Embryos were cultured in vitro and transferred to recipients as described¹⁸. Genome editing was performed at the in vivo Research Facility of the CECAD Research Center, University of Cologne, Germany.

Genetic validation of Knock-In

For a PCR to validate the integration of our construct into the Rosa26 locus as described by Chu et. al., we used the primers SAR CCTGGACTACTGCGCCCTACAGA und R26F3 CTGCCCCGAGCGGAAACGCCACTGA C¹⁶. The PCR was carried out using the Herculanse II Fusion DNA Polymerase (Agilent Technologies, #600675) resulting in a ~1.3 kb fragment. After successful integration validation, the genetic locus was further analyzed by Cergentis. Following their protocol (www.cergentis.com) bone marrow of heterozygous mice was prepared and analyzed. Two sets of primers (see Table 1) were used for targeted Locus Amplification (TLA) sequencing.

Mice

Animals were derived and maintained in the CECAD Research Center, University of Cologne, Germany, in individually ventilated cages (Greenline GM500; Tecniplast). Mice were kept at 22 °C (± 2 °C) and a relative humidity of 55% (± 5%) under 12-h light cycle on sterilized bedding (Aspen wood, Abedd, Germany) and with access to sterilized commercial pelleted diet (Ssniff Spezialdiäten GmbH) and acidified water ad libitum. The microbiological status was examined as recommended by the Federation of European Laboratory Animal Science Associations (FELASA) and the mice were free of all listed agents including opportunists. Mice (C57BL/6N) of both sexes at the age of 4 weeks and 62 weeks were used. Generated C57BL/6N-Gt(ROSA)26Sor^{em333}(CAG-mScarlet-I)^{Cecad}/Cecad (short: Lifeact.mScarlet-I) were crossed to podocin-Cre (MGI: 3586451) to achieve expression in all podocytes (Pod^{Lifeact.mScarlet-I}). Inducible expression was obtained by using the tamoxifen responsive podocin-iCreERT2 (MGI: 4819592) to generate iPod^{Lifeact.mScarlet-I}^{19,20}. Cre negative animals (Pod^{wt}) were used as control. For phenotypic analysis, 3 mice per age with the genotype Pod^{Lifeact.mScarlet-I} were used. As control 2 Pod^{wt} per age group were used. No animals were excluded, no randomization was performed. Potential confounders were not controlled and blinding of the study was not performed. N equals the number of mice if not stated otherwise. For measurement of SD length/area, 5 glomeruli per animal were imaged and analyzed. Additionally, Pod^{Lifeact.mScarlet-I} mice were crossed with R26.GCaMP3 mice (MGI: 5314900), resulting in a Pod^{Lifeact.mScarlet-I}/GCaMP3 line, expressing the green fluorescent protein GFP (GCaMP3), which we used as a

Primer set	Name	Direction	Binding position	Sequence
1	PURO	RV	4,058	GGCCTTCCATCTGTTGCTG
	PURO	FW	4,247	CCCTTCTACGAGCGGCTC
2	mScarlet-I	RV	5,096	GCCCTGGAGCCGTACATG
	mScarlet-I	FW	5,537	ACAGTACGAACGCTCCGA

Table 1. TLA sequencing primers.

marker for podocyte cell bodies²¹. Animal experiments complied with ARRIVE guidelines²². For the preparation of AKS and STED tissue harvest, mice were euthanized by cervical dislocation. At the end of in vivo imaging experiments mice were euthanized by cervical dislocation, while still under anesthesia.

Genotyping protocol

For genotyping, we used skin biopsies obtained by mouse ear tagging. DNA was extracted using the HotSHOT protocol and amplified using REDTaq ReadyMix (Sigma-Aldrich)²³. For R26.LifeAct.mScarlet-I mice the following protocol was used: amplification of the Rosa26 wildtype allele using the forward primer GGAGTGTTCGAATACCTTTCTGGGAGTTC and reverse primer CGAGGCGGATCACAAGCAATA results in a ~350 bp band. The Rosa26 transgenic locus was amplified using the forward primer GGGGAGGATTGGGAAGACAA together with CGAGGCGGATCACAAGCAATA as the reverse primer resulting in a 560 bp band. Genotyping of R26.GCaMP3 mice was performed as previously published²⁴. Genotyping for Podocin-Cre and podocin-iCreERT2 was performed as previously published^{19,20}.

Analysis of proteinuria and creatinine

Proteinuria was evaluated by measuring albumin content in urine samples. In short, 2 µl of urine sample were mixed with 8 µl H₂O and 2 µl 6×Laemmli-loading dye. Samples were boiled (5 min, 95 °C) and loaded onto a 10% SDS gel. As a reference for protein content 1 µg and 10 µg bovine serum albumin (BSA) were used. After gel electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualize albumin bands. For a urinary albumin ELISA, a 96-well plate was covered with an α-mouse albumin antibody (1:10.000) for 1 h at RT (A90-134A, Bethyl Laboratories). The plate was washed 5 times and incubated with a blocking solution for 30 min followed by further 5 washing steps. An albumin standard was prepared for a reference curve ranging from 0 to 625 ng/ml. Urines were diluted to a concentration of around 150 ng/ml. Standard and urine samples were added to the plate and removed after 1 h. For detection, an HRP-coupled goat α-mouse antibody (1:25.000) was used (# A90-134P, Bethyl Laboratories). Ultimately, the absorbance at 450 nm was measured on a plate reader (Perkin Elmer).

Urinary creatinine was determined using a commercially available kit (Creatinine (urinary) Colorimetric Assay Kit, Item No. 500701, Cayman Chemical).

Tamoxifen induction

LifeAct.mScarlet-I^{fl/wt}ERT2.Pod:cre^{tg/wt} (iPod^{LifeAct.mScarlet-I}) mice were induced with Tamoxifen (0.1 mg/g body weight) in sesame oil via oral gavage at the age of 3 weeks. Control animals received sesame oil via oral gavage. Mice were monitored for weight loss and sacrificed 5 days after induction to either prepare acute kidney slices or used for in vivo imaging.

In vivo imaging

Preparation and in vivo imaging were performed as previously published²⁵. Before surgery mice received buprenorphine (RB Pharmaceuticals Limited, 0.1 mg/g BW) as an analgesic drug and were anesthetized with isoflurane (Piramal Critical Care, #9714675, 1.5–5%). A 1 cm long incision in the midline of the neck was made and the right carotid artery was dissected. The artery was ligated cranially and clamped caudally. A small incision was made, and a catheter (PE-10) was inserted and fixed with sutures. Next, the left kidney was exteriorized through a small incision above the kidney and immobilized with a custom-build kidney holder (Medres). A coverslip was placed on the kidney with minimum pressure. A 70-kDa fluorescein coupled dextran (D1823, Invitrogen) was administered through the catheter to label the blood circulation. The body temperature was maintained with a heating system (Medres). In vivo imaging was carried out using an upright multiphoton microscope (TCS SP8 MP-OPO; Leica Microsystems) and a 25× water immersion objective (numerical aperture: 0.95). The multiphoton laser (Chameleon Vision II; Coherent) was tuned to 940 nm and images were recorded using non-descanned hybrid detectors and a FITC/TRITC (525/50) filter set. Stacks of glomeruli were recorded with a 2 µm step size. Laser power was adjusted to the brightness of the observed podocytes to minimize saturation.

Preparation and imaging of acute kidney slices

Fresh mouse kidneys were prepared as previously described²⁶. Shortly, kidneys were embedded in low-melting agarose (4%) and cut into 300 µm slices using a vibratome (VT1200 S, Leica). Slices were maintained in Krebs–Henseleit–Buffer carbonated with 95% O₂/5% CO₂²⁷. For imaging, single slices were transferred into an imaging dish and stabilized with a slice anchor (Warner Instruments) to reduce tissue drifting. Multiphoton imaging was performed with a TCS SP8 MP-OPO microscope (Leica Microsystems) with a Chameleon Vision II laser (Coherent) at 940 nm.

STED sample preparation and imaging

Immunofluorescent labeling was conducted as previously described¹⁴. Briefly, 4% PFA-fixed kidney tissue was immersed in a hydrogel solution. The gel was polymerized for 3 h at 37 °C. Then, the tissue was embedded in 3% agarose and cut into 200 µm thick slices using a vibratome (VT1200 S, Leica). The slices were optically cleared overnight, followed by incubation with target protein antibodies (see Table 2). Confocal and STED images of fixed slices were acquired with a Leica TCS SP8 gSTED 3X equipped with a white light laser (WLL) and a 775 nm STED depletion laser (Leica Microsystems).

Antibody	Dilution	Cat no. and company
Synaptopodin (SE-19)	1:200	S9442-200UL, Sigma
tdTomato	1:200	AB8181-200, Sicgen
Human Nephrin Antibody	1:100	AF4269, R&D Systems
RFP	1:200	600-401-379, Rockland
Anti-Tubulin, Acetylated	1:200	T6793-100UL, Sigma
Vimentin (D21H3) XP (Alexa488 conjugated)	1:100	9854S, Cell Signaling
GFP (Alexa647 conjugated)	1:100	A-31852, Invitrogen
FluoTag*-X2 anti-mScarlet-I (AbberiorStar635P conjugated)	1:50	N1302-Ab635P-L, NanoTag Biotechnologies
Secondary antibody		
Donkey anti-rabbit Alexa488	1:100	Jackson ImmunoResearch
Donkey anti-goat AbberiorStar635P	1:100	Jackson Immunoresearch, coupled in house
Donkey anti-goat Atto594	1:100	Jackson Immunoresearch, coupled in house
Donkey anti-rabbit Atto594	1:100	Jackson Immunoresearch, coupled in house
Donkey anti-rabbit AbberiorStar635P	1:100	Jackson Immunoresearch, coupled in house
Donkey anti-mouse Alexa488	1:100	Jackson ImmunoResearch

Table 2. Antibodies used for immunofluorescent stainings.

Image processing

Images were processed using FIJI and Omero.figure web application. Movie of 3D rendering of z-stack was created using Imaris.

Statistical analysis

For statistical analysis of data, the software Graph Pad Prism (Version 10.1.2) was used. We performed an ordinary two-way ANOVA with multiple comparisons for all performed tests in Fig. 2. No data points were excluded. ns $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

Results

To generate a conditional Lifeact.mScarlet-I mouse line, the desired sequence was cloned into the ROSA targeting vector pR26 CAG AsiSI/MluI¹⁶. The plasmid was then targeted with CRISPR/Cas9 genome editing to the Gt(ROSA)26Sor locus as previously described (Fig. 1a)¹⁶. Integration PCR performed on the 9 founder animals (#1–9) resulted in one positive result (#7) with a band at 1.3 kB (Fig. 1b, Suppl. Fig. S5). Offspring of this mouse were then used to establish the mouse line. We performed TLA and sequencing of F₁ mice to verify the correct sequence and integration of the insert as well as exclude unspecific vector integration. TLA sequencing showed correct integration in the Gt(ROSA)26Sor locus on chromosome 6 between exons 1 and 2 without additional integration sites in the entire genome (Fig. 1c). Apart from the notoriously complex CAG promoter sequence, TLA sequencing also confirmed the sequence of the entire insert without any mutation (Suppl. Fig. S1).

We then used Pod-Cre (Pod^{Lifeact.mScarlet-I}) or tamoxifen inducible Pod-ERT2-Cre (iPod^{Lifeact.mScarlet-I}) mice to express Lifeact.mScarlet-I specifically in podocytes (Fig. 1d). Lifeact.mScarlet-I expression in heterozygous Pod^{Lifeact.mScarlet-I} animals resulted in bright podocyte specific fluorescence in acute kidney slices (AKS) (Fig. 1e). In fixed tissue Lifeact.mScarlet-I completely co-localized with the actin binding protein synaptopodin (Fig. 1f).

To exclude the occurrence of adverse effects of Lifeact.mScarlet-I expression on kidney function due to binding to the actin cytoskeleton we investigated young mice at the age of 4-weeks and aged mice at 62-weeks of age. We therefore compared heterozygous Pod^{Lifeact.mScarlet-I} animals to Cre negative littermates (Pod^{wt}).

Spot urine samples revealed no albuminuria in young mice, while aged Pod^{Lifeact.mScarlet-I} animals showed mild proteinuria in their urine samples compared to same-age Pod^{wt} littermates (Fig. 2a, Suppl. Fig. S6). This is also reflected by the increased albumin creatinine ratio (ACR) in 62-week old Pod^{Lifeact.mScarlet-I} mice, while 4-week old mice of both genotypes and 62-week old Pod^{wt} had low ACRs (Fig. 2b). However, serum creatinine levels in young Pod^{wt} and Pod^{Lifeact.mScarlet-I} mice were higher compared to the aged mice of both genotypes (Suppl. Fig. S2). To evaluate the effects of Lifeact.mScarlet-I expression on SD structure, we performed stimulated emission depletion (STED) microscopy to visualize nephrin expression at the SD (Fig. 2c). Young Pod^{Lifeact.mScarlet-I} mice showed a small reduction of the SD length/area compared to Pod^{wt} animals (Fig. 2d). Old Pod^{Lifeact.mScarlet-I} mice presented with a significant further reduction in SD length/area compared to old Pod^{wt} animals, indicating a mild effect of LifeAct.mScarlet-I expression on SD integrity during aging (Fig. 2d).

Subsequently, we performed intravital imaging of the kidney to evaluate the Lifeact expression pattern in 4-week old Pod^{Lifeact.mScarlet-I} animals in vivo. Glomerular blood flow was labeled by injecting a FITC conjugated 70 kDa dextrane. A video of the acquired z-stack shows capillary blood flow (Suppl. Video V1). Pod^{Lifeact.mScarlet-I} mice showed a homogenous expression of Lifeact.mScarlet-I in all podocytes as depicted in the maximum intensity projection (MIP) (Fig. 3a). Single plane images show strong Lifeact.mScarlet-I fluorescence close to the capillary which indicates labeling of FPs (Fig. 3a' and a''). Next, we prepared acute kidney slices (AKS) to establish Lifeact.mScarlet-I as an ex vivo tool to study the cytoskeleton²⁶. Comparable to podocyte in vivo imaging, glomeruli of Pod^{Lifeact.mScarlet-I} mice showed homogenous expression of Lifeact.mScarlet-I adjacent to

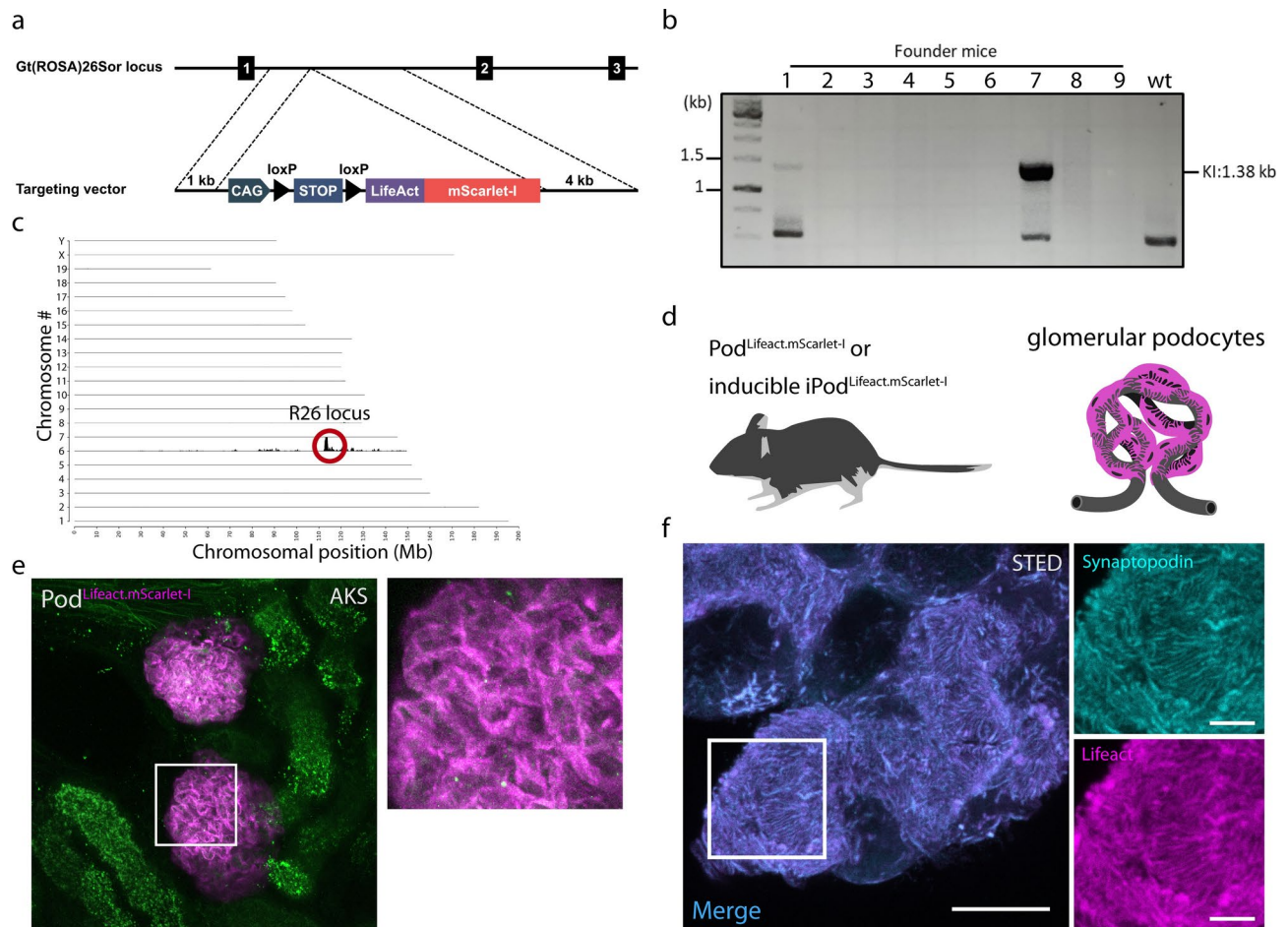


Fig. 1. Generation of Lifeact.mScarlet-I mice to label filamentous actin. **(a)** Targeting strategy of the ROSA26 locus for site-specific integration of R26 LSL Lifeact.mScarlet-I to mediate Cre dependent expression. **(b)** Integration PCR of different founder mice to validate successful integration in the F₀ generation. **(c)** TLA sequence coverage across the mouse genome using primer set 2. The chromosomes are indicated on the y-axis, the chromosomal position on the x-axis. Identified integration site is encircled in red. **(d)** The Lifeact.mScarlet-I construct was expressed in glomerular podocytes using podocin-cre (Pod^{Lifeact.mScarlet-I}) or tamoxifen inducible podocin-Cre-ERT2 (iPod^{Lifeact.mScarlet-I}). Created with BioRender. **(e)** Maximum intensity projection (MIP) image of Lifeact.mScarlet-I fluorescence in podocytes in murine acute kidney slice (AKS). **(f)** Representative MIP STED microscopy images of Pod^{Lifeact.mScarlet-I} glomerular podocytes co-stained with synaptopodin. Annotation: KI Knock-In, TLA Targeted locus amplification, MIP maximum intensity projection, AKS Acute kidney slice. Scale bar—10 μm, zoom in—3 μm.

the capillary (Fig. 3b-b''). However, the resolution of multiphoton microscopy was too low to visualize single podocyte FPs in an in vivo or ex vivo setting using this approach. Therefore, we used the Lifeact.mScarlet-I mouse model to visualize the actin cytoskeleton in FPs by super-resolution microscopy. Staining of mScarlet-I using an α-RFP antibody showed a homogenous staining pattern in Pod^{Lifeact.mScarlet-I} mice. The MIP shows intense labeling of the capillaries and minimal labeling of the cytoplasm within the cell body (Fig. 3c-c'). STED images of the capillary loops show a densely packed layer of FPs which covers the entire surface in a regular pattern (Fig. 3c''). The SD is visible as an unstained line between single FPs. 3D rendering of a z-stack revealed an actin distribution with pronounced ridges between single FPs wrapping around the glomerular capillary (Suppl. Video V2).

We validated the specificity of RFP or tdTomato antibodies for mScarlet-I by co-staining with an anti-mScarlet-I nanobody (Suppl. Fig. S3). Even though the nanobody penetrated deeper into the tissue, high concentrations were needed to obtain a similar staining intensity.

Next, we prepared AKS from Pod^{Lifeact.mScarlet-I} mice to test the possibility of visualizing dynamic actin reorganization. We, therefore, incubated AKS of Pod^{Lifeact.mScarlet-I} with increasing doses of methyl-β-cyclodextrin (MβCD). MβCD depletes cholesterol from plasma membranes and disrupts lipid rafts and thus the SD complex. Figure 4a shows a representative image of a control glomerulus without MβCD with a normal distribution of Lifeact.mScarlet-I. After incubation with 20 mM MβCD for 4 h the regular actin pattern is partially disrupted (Fig. 4b) and actin blebbing is visible in AKS (Fig. 4c', arrow). After fixation, the observed actin blebs remained and a reduction of the continuous coverage of the capillary with actin cables became evident (Fig. 4d', arrow).

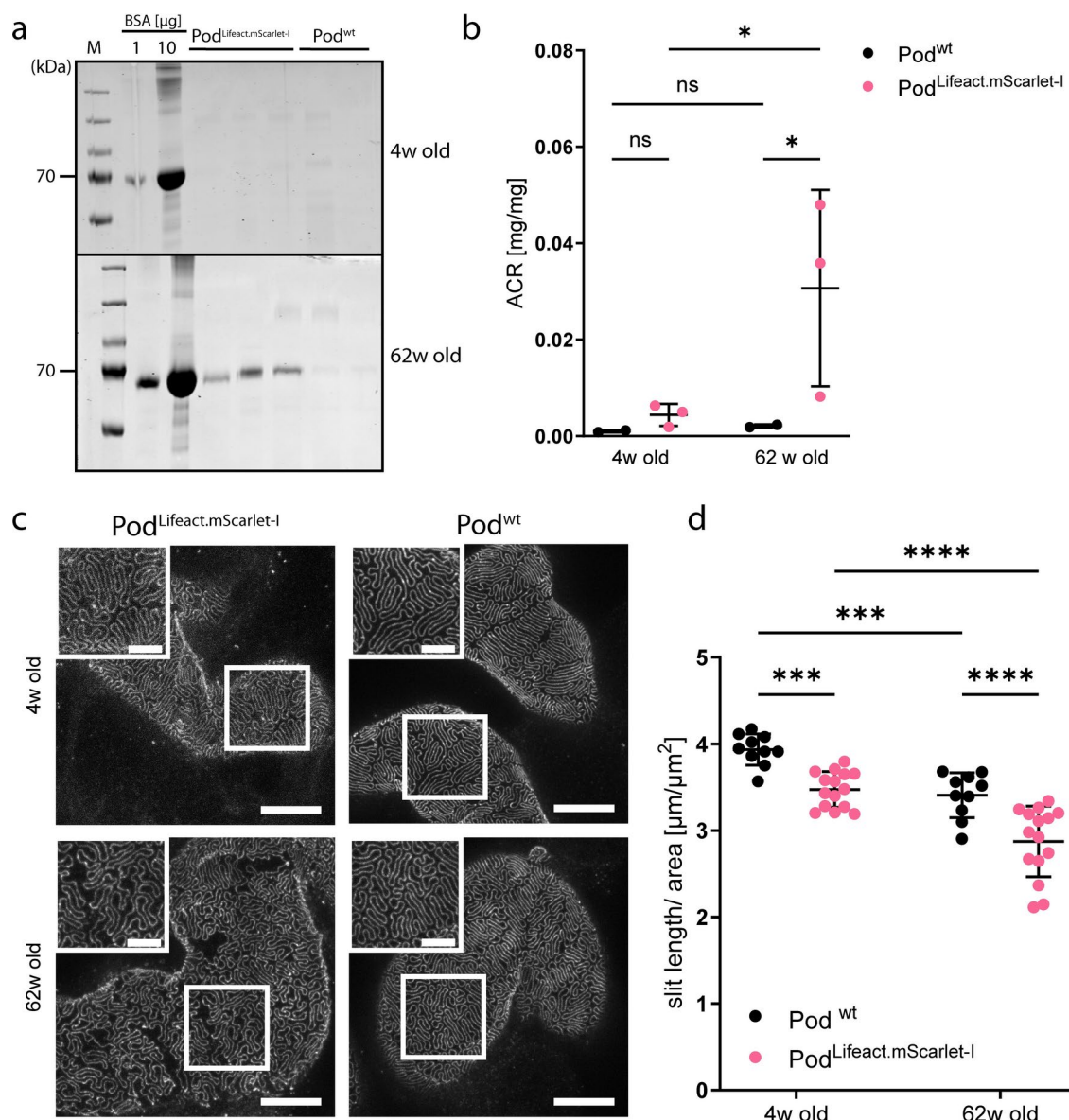


Fig. 2. Phenotypic analysis of young and aged Lifeact.mScarlet-I mice. **(a)** Coomassie urine analysis of 4- and 62-week old Pod^{Lifeact.mScarlet-I} (n = 3 per age) and Pod^{wt} (n = 2 per age) mice. 1 and 10 μ g bovine serum albumin (BSA) was used as a standard. **(b)** Urinary albumin/creatinine ratio from spot urine samples of 4- and 62-week old Pod^{Lifeact.mScarlet-I} (4-weeks: n = 3; 62 weeks: n = 3) and Pod^{wt} (4-weeks: n = 2; 62 weeks: n = 2) mice. **(c)** Representative images of nephrin expression in 4- and 62-week old Pod^{Lifeact.mScarlet-I} and Pod^{wt} mice using STED microscopy. **(d)** Slit diaphragm length/area measurements of 4- and 62-week old Pod^{Lifeact.mScarlet-I} (n = 15 glomeruli of three mice each) and 4- and 62-week old Pod^{wt} (n = 10 glomeruli of two mice each). Scale bar—5 μ m, zoom in—2 μ m. Statistical analysis: Ordinary two-way ANOVA with multiple comparisons. ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Noticeably cells detached from the capillary surface during processing of the sample indicated by asterisks, which was not the case in control animals (Fig. 4d). In areas of podocyte detachment the SD pattern was completely disrupted.

To enhance contrast during imaging we used a tamoxifen inducible Pod-ERT2-Cre (iPod^{Lifeact.mScarlet-I}) mouse line to achieve Lifeact.mScarlet-I expression only in a subset of podocytes. iPod^{Lifeact.mScarlet-I} mice (0.1 mg/g tamoxifen, 5 days before imaging) revealed partial expression of the reporter. Thus, parts of capillary loops within the glomerulus are covered by podocytes without fluorescent labeling (Fig. 5a-a''). The same was true for AKS prepared from iPod^{Lifeact.mScarlet-I} animals (Fig. 5b-b''). STED imaging of iPod^{Lifeact.mScarlet-I} shows labeling of single podocytes. The high contrast allows for visualization of an individual podocyte with its foot processes (Fig. 5c-c''). Control iPod^{Lifeact.mScarlet-I} animals without tamoxifen induction showed only rarely Lifeact.mScarlet-I positive cells as a sign of a slight leakiness of the inducible promoter (Suppl. Fig. S4).

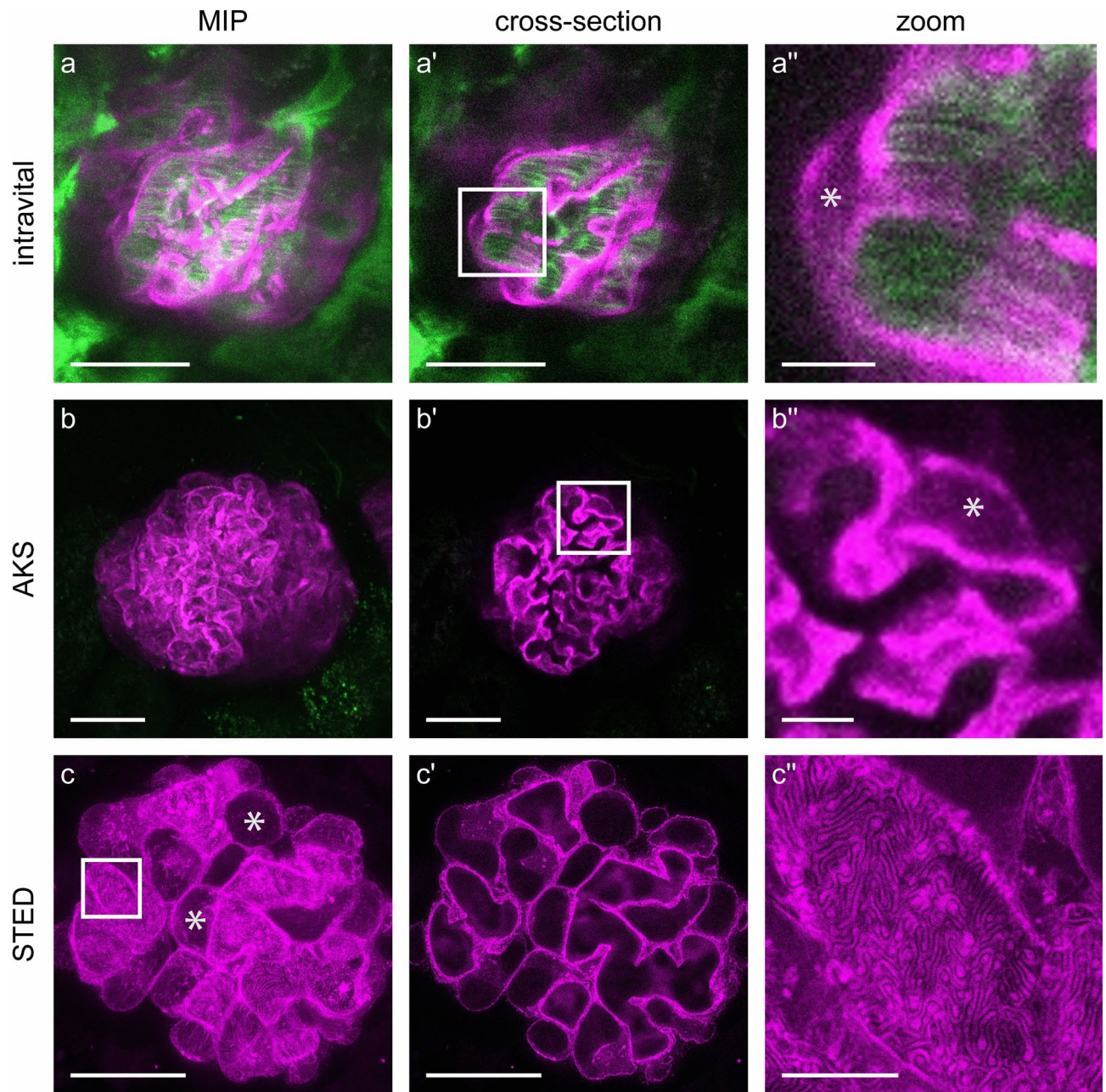


Fig. 3. Homogenous expression of Lifeact.mScarlet-I in glomerular podocytes. **(a)** Representative images of Lifeact.mScarlet-I fluorescence in podocytes imaged by intravital microscopy using a multiphoton microscope (magenta). Blood flow is visualized by injection of 75-kDa FITC coupled dextran (green). **(b)** Murine AKS of Pod^{Lifeact.mScarlet-I} animals imaged with multiphoton microscopy. **(c)** Immunofluorescently labeled Lifeact.mScarlet-I tissue section imaged with STED microscopy. Asterisks indicate podocyte cell bodies. Scale bars—25 μ m, zoom—5 μ m; MIP maximum intensity projection; AKS Acute kidney slice.

Additionally, we co-stained Lifeact.mScarlet-I with marker proteins relevant for podocyte ultrastructure, such as acetylated tubulin, vimentin and nephrin. Their localization in podocytes is depicted in a schematic overview (Fig. 6a). We first used a transgenic mouse expressing cytosolic GFP (Pod^{Lifeact.mScarlet-I/GCaMP3}) in addition to Lifeact.mScarlet-I in podocytes. GFP was evenly distributed in the cytosol, while Lifeact.mScarlet-I was most prominent in FPs (Fig. 6b-b''). Tubulin is the main cytoskeletal component of primary processes in podocytes and we were interested in the localization of this cytoskeletal protein with regard to actin fibers. Co-staining of acetylated tubulin and Lifeact.mScarlet-I in iPod^{Lifeact.mScarlet-I} kidney tissue clearly shows that tubulin is exclusively expressed in primary processes, while actin is located in FPs (Fig. 6c-c''). Vimentin, a type of intermediate filament, was mostly present at the base of the cell body and in primary processes, while Lifeact-mScarlet-I staining was mostly limited to the FPs (Fig. 6d-d''). The co-staining with nephrin revealed the localization of the slit diaphragm along the actin fingers in FPs (Fig. 6e-e''). Orthogonal views of a capillary

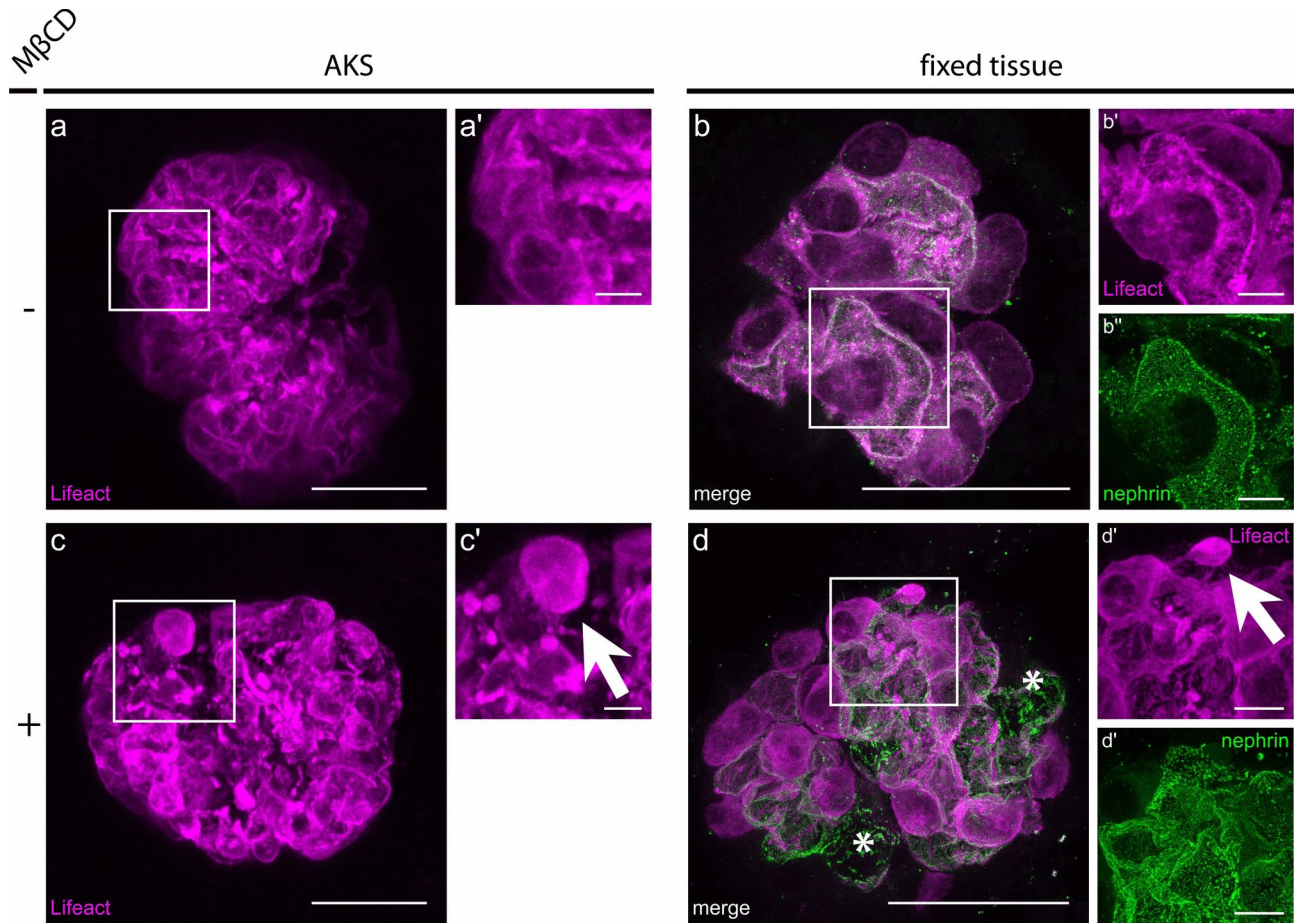


Fig. 4. Depletion of lipid rafts leads to actin disruption in Pod^{Lifeact.mScarlet-I} acute murine kidney slices. (a) Representative image of Lifeact.mScarlet-I pattern (magenta) in control conditions using multiphoton microscopy. (b) Representative images of Lifeact.mScarlet-I (magenta) and nephrin pattern (green) in control conditions in fixed tissue using STED microscopy. (c) Representative image of a glomerulus after incubation with 20 mM MβCD for 4 h imaged with multiphoton microscopy. Arrow in zoom-in indicates actin blebbing (c'). (d) Representative image of a Lifeact.mScarlet-I (magenta) and nephrin pattern (green) after incubation with 20 mM MβCD for 4 h in fixed tissue imaged with STED microscopy. Arrow in zoom-in indicates actin blebbing, asterisk indicates areas with detached podocytes. Scale bars—25 μm, zoom—5 μm. MβCD Methyl-β-cyclodextrine.

wall cross-section show an alternating nephrin-Lifeact.mScarlet-I-nephrin pattern followed by an empty space of an unlabeled FP (Fig. 6f, XY and XZ view).

Taken together, this new Lifeact.mScarlet-I mouse line represents a powerful tool to visualize the actin cytoskeleton in podocytes in vivo, ex vivo and in STED microscopy in a resolution previously only obtainable by electron microscopy.

Discussion

Here we present a novel mouse model for tissue-specific expression of Lifeact.mScarlet-I. The reporter is bright, photo stable and suitable for intravital and ex vivo imaging to monitor actin structures. Expression was limited to Cre expressing cells, as we did not observe mScarlet-I positive cells within other kidney structures such as tubular cells or endothelial cells. It is therefore plausible that expression of Lifeact.mScarlet-I is also specific using other Cre drivers. Thus, our line is a promising universal tool to visualize F-actin in any tissue desired.

TLA sequencing revealed the correct insertion of a single copy of the Lifeact.mScarlet-I construct into the Gt(ROSA)26Sor locus with no mutations, inversions, or unspecific integration. Phenotypic analysis of young Pod^{Lifeact.mScarlet-I} and Pod^{wt} animals for general kidney function showed no proteinuria, but higher serum creatinine levels than in older animals. This increase observed in both Pod^{wt} and Pod^{Lifeact.mScarlet-I} genotypes seems to be independent of Cre activity and could be due to diet biases and measurement errors²⁸. Analysis of SD length/area morphology in young 4-week-old animals showed a small but significant reduction between Pod^{Lifeact.mScarlet-I} compared to Pod^{wt}. This decrease is not typically associated with any changes in proteinuria or histology. Older Pod^{Lifeact.mScarlet-I} animals developed mild proteinuria that correlates with a further reduction of the SD length. This may be caused by interference of Lifeact binding with physiological actin turnover. Intravital

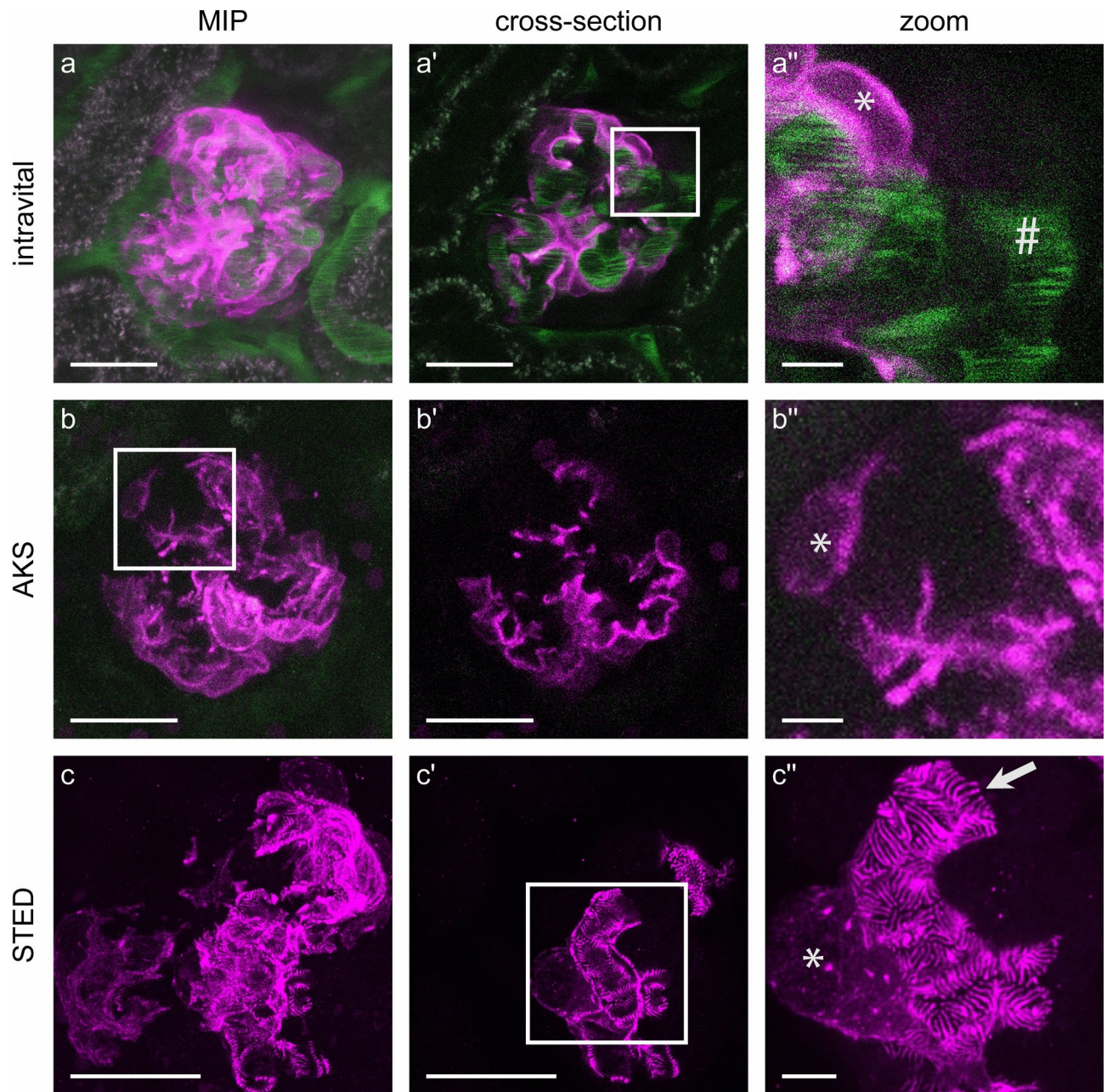


Fig. 5. Tamoxifen induced partial expression of Lifeact.mScarlet-I in glomerular podocytes. (a) Representative images of Lifeact.mScarlet-I fluorescence (magenta) in podocytes of an $iPod^{Lifeact.mScarlet-I}$ mouse imaged by intravital microscopy using a multiphoton microscope. Blood flow is visualized by injection of 75-kDa FITC coupled dextran (green). (b) Murine AKS of an $iPod^{Lifeact.mScarlet-I}$ animal imaged with multiphoton microscopy. (c) Immunofluorescently labeled Lifeact.mScarlet-I tissue section imaged with STED microscopy. All animals received a single dose of tamoxifen via oral gavage (0.1 mg/g body weight) and were sacrificed 5 days later. Asterisks indicate podocyte cell bodies. Hashtag #—glomerular capillary; Arrow—single podocyte foot processes; Scale bars—25 μ m, zoom—5 μ m; MIP maximum intensity projection; AKS Acute kidney slice.

imaging experiments are only performed with young animals, and our data show that heterozygous Lifeact.mScarlet-I expression is not affecting kidney function at this age. Due to the bright fluorescence of mScarlet-I, homozygous expression is not necessary for sufficient fluorescence intensity. The effect of $Pod^{Lifeact.mScarlet-I}$ expression on the kidney filter became even more pronounced with age. In line with our results, recently published mouse models such as the beta-actin-GFP mouse line also showed the development of early onset proteinuria and glomerulosclerosis upon aging²⁹.

In $Pod^{Lifeact.mScarlet-I}$ animals we observed a homogeneous labeling of the podocyte actin cytoskeleton, which is mainly localized in the foot processes and barely in the cell body. The expression pattern of mScarlet-I co-localized with synaptopodin, an actin-binding protein, which is commonly used to indirectly label actin in podocytes in

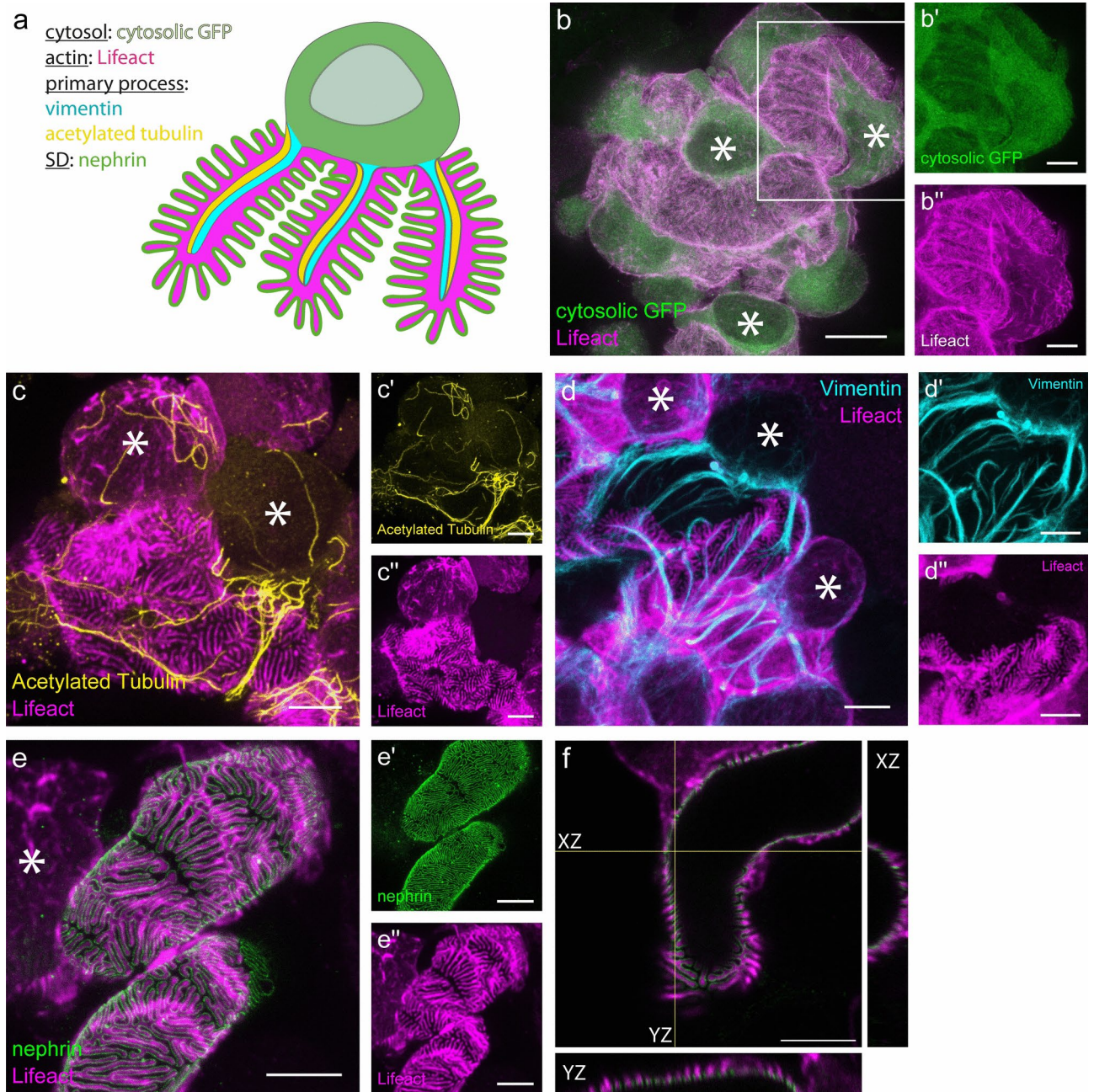


Fig. 6. Immunofluorescent stainings of podocyte cytoskeletal components. (a) Schematic podocyte with color indicating which cellular compartments were stained in the panels (b–f). (b) Staining of a cytosolic GFP (green) and Lifeact (magenta) in a Pod^{Lifeact.mScarlet-1} mouse glomerulus. Single colors are shown for GFP in b' and Lifeact in b''. (c) Merged image of a single podocyte of an iPod^{Lifeact.mScarlet-1} mouse stained for acetylated tubulin (yellow) and Lifeact (magenta). Single channels are shown for acetylated tubulin in c' and Lifeact in c''. (d) Labelling of intermediate filaments by vimentin staining (cyan) and Lifeact (magenta) in a mouse glomerulus of an iPod^{Lifeact.mScarlet-1} animal. Single channels are shown for vimentin in d' and Lifeact in d''. (e) Merged STED image of nephrin (green) and Lifeact (magenta) of an iPod^{Lifeact.mScarlet-1} mouse. Single colors are shown for nephrin in e' and Lifeact in e''. (f) Orthogonal views of foot processes covered glomerular capillary surface with Lifeact (magenta) and nephrin (green) of an iPod^{Lifeact.mScarlet-1} mouse. All iPod^{Lifeact.mScarlet-1} animals received a single dose of tamoxifen via oral gavage (0.1 mg/g body weight) and were sacrificed 5 days later. All images are maximum intensity projections except (f). Asterisks mark podocyte cell bodies. All scale bars—5 μm, except (b)—10 μm.

fixed tissue³⁰. However, labeling of actin fibers or synaptopodin in all podocytes impaired visualization of single FPs. We therefore used a tamoxifen inducible mouse model to overcome this issue. In iPod^{Lifect.mScarlet-I} animals the application of a single low dose tamoxifen achieved visualization of single mScarlet-I positive podocytes 5 days later. In these podocytes, the fine and complex structure of FPs could be visualized in detail. Control animals that received only sesame oil did show a low number of mScarlet-I positive cells, indicating minor leakiness of the Pod-ERT2-Cre line.

The gold standard to visualize podocyte cytoskeletal structures has been electron microscopy³¹. Even though electron microscopy can achieve higher resolution than STED imaging, serial sectioning techniques are extremely time consuming and do not allow for co-staining of additional target proteins. Furthermore, using super-resolution imaging, multiple regions within a sample can be analyzed to give a comprehensive understanding of changes in FP morphology.

Recently, podocyte actin structure has been imaged by Airy scan microscopy using phalloidin staining of isolated glomeruli³². However, the method requires extraction of glomeruli, which can result in a high number of dead cells and might affect the delicate structure of FPs²⁵. Using our novel mouse model conventional confocal imaging of cleared kidney tissue, allowed for visualization of podocyte cell body and primary processes and general actin distribution. However, the resolution was not sufficient to visualize the SD. Additional expansion of the tissue, could be sufficient to circumvent the need for super-resolution microscopy^{33,34}.

To demonstrate that our mouse model is capable of visualizing actin changes, we incubated AKS with the cholesterol-depleting agent M β CD. Before treatment, actin was mainly located within the FPs and showed an even distribution. After incubation, rearrangements of the actin cytoskeleton were visible using multiphoton microscopy. Actin was localized more in the cell body and showed actin blebbing³⁵. The effect of M β CD was even more striking in super-resolution microscopy. The fine FPs were no longer covering the capillary completely. The subsequent loss of podocytes destroyed the SD partially.

Combining different disease models such as nephrotoxic serum nephritis or adriamycin nephropathy with the Pod^{Lifect.mScarlet-I} and iPod^{Lifect.mScarlet-I} mouse model will allow us to study the effects on the podocyte actin cytoskeleton in vivo and ex vivo upon podocyte injury. Even the highest possible resolution obtained with our multiphoton imaging setup, limits its application for studying fine structures like actin. Combining minimal labeling using iPod^{Lifect.mScarlet-I} mice and implanting an optical abdominal window placed above the kidney, would allow intravital imaging of the same region within the kidney over time³⁶. We therefore see the scope of application for intravital multiphoton microscopy in visualization of the actin cytoskeleton during disease progression, while super-resolution microscopy is limited to fixed and cleared samples of a single time point.

Furthermore, our new mouse models can be combined with genetic disease models including mutations of the SD proteins to understand the structural rearrangements resulting in podocyte FP effacement and focal segmental glomerulosclerosis. Finally, due to its red fluorescence, Lifect.mScarlet-I is compatible with commonly used green fluorescent reporters such as GCaMPs. The combination of both reporters would allow us to understand the interplay between calcium signaling and actin in vivo.

In conclusion, due to its monomeric nature and high fluorescence Lifect.mScarlet-I is the ideal reporter protein to label actin structures with minimal side effects in podocytes in vivo, ex vivo and in fixed tissue. It will allow us to understand how actin rearranges during disease, especially during FP effacement.

Data availability

The datasets generated during and/or analysed during the current study are available in the Figshare repository: <https://doi.org/10.6084/m9.figshare.26797678.v4>. All the sequencing reads generated from TLA sequencing conducted by Cergentis are available in the NCBI SRA: <https://www.ncbi.nlm.nih.gov/sra/PRJNA1214545>; accession SRX27437702 and SRX27437701. The BioProject accession number is PRJNA1214545.

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Author contributions

Multiphoton and STED imaging experiments were designed by J.B.-L. and M.J.H.. S.E.T. and B.Z. generated the mouse model. Experiments were performed by E.W., J.B.-L.. B.S. generated the schematic overview. The manuscript was written by E.W., J.B.-L., N.R. and M.J.H.. T.B., R.W.-S., D.U.-J. and M.J.H. provided guidance and resources.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

All animal protocols were performed in compliance with the European, national and institutional guidelines and approved by the State Office of North Rhine-Westphalia, Department of Nature, Environment and Consumer Protection (LANUV NRW, Germany; animal study protocol AZ 81-02.04.2019.A335, AZ 81-02.04.2018.A351, AZ 81-02.04.2023.A301). Animal experiments are reported in accordance with ARRIVE guidelines²¹.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-96822-w>.

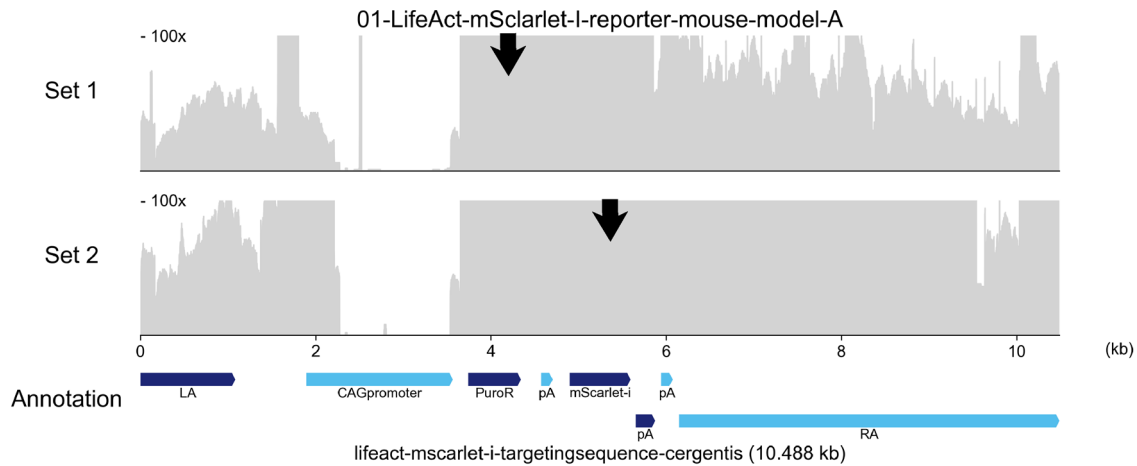
Correspondence and requests for materials should be addressed to M.J.H.

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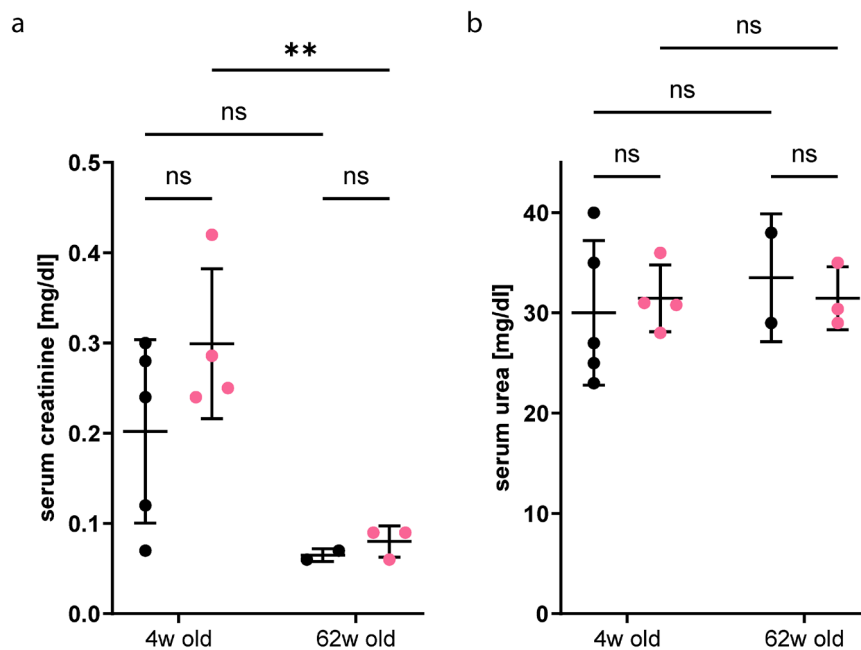
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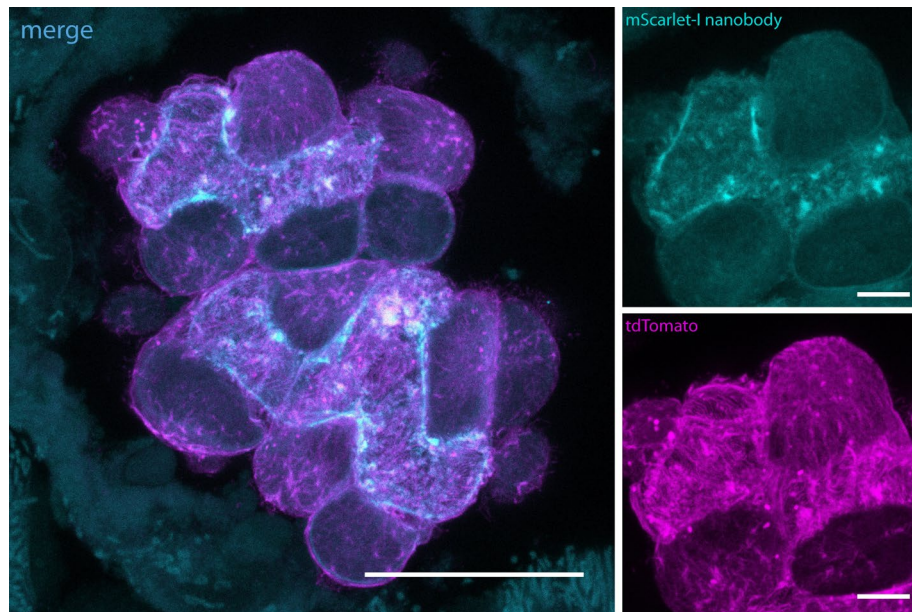
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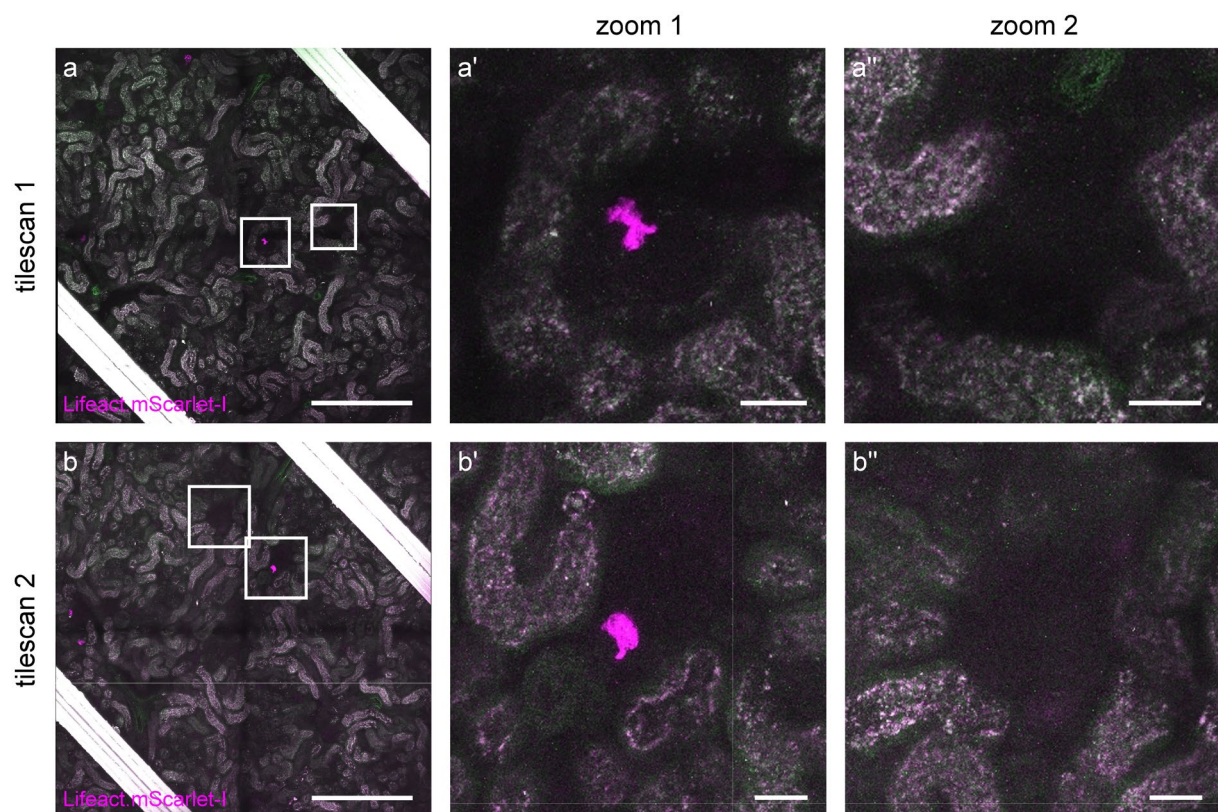
Supplementary Figure S1. Targeted locus amplification sequencing for validation of correct Lifeact.mScarlet-I integration into the R26 locus. NGS sequencing coverage (in grey) across the Lifeact.mScarlet-I vector. Black arrows indicate the primer binding location. The annotated vector map is shown at the bottom. Y-axes are limited to 100x. Annotation: LA – Left homology arm, PuroR – Puromycin Resistance, pA – Poly A tail, RA – Right homology arm.



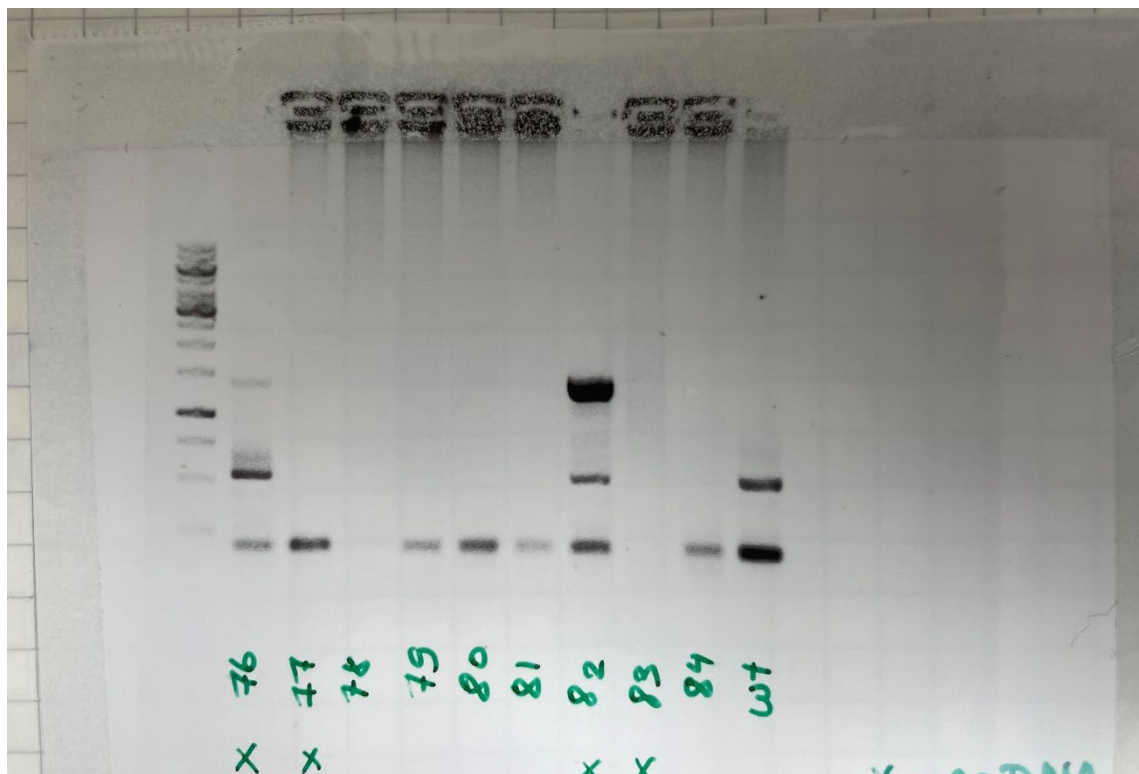
Supplementary Figure S2. Serum urea and creatinine levels measured in young and aged Pod^{Lifeact.mScarlet-I} mice. (a) Serum creatinine levels of young and aged Pod^{Lifeact.mScarlet-I} mice. (b) Serum urea levels of young and aged Pod^{Lifeact.mScarlet-I} mice. In both measurements the following animals were analyzed: Pod^{Lifeact.mScarlet-I} 4-weeks: n=4; 62 weeks: n=3 and Pod^{wt}: 4-weeks: n=5; 62 weeks n=2. Statistical analysis: Ordinary two-way ANOVA with multiple comparisons. ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001, **** p ≤ 0.0001.



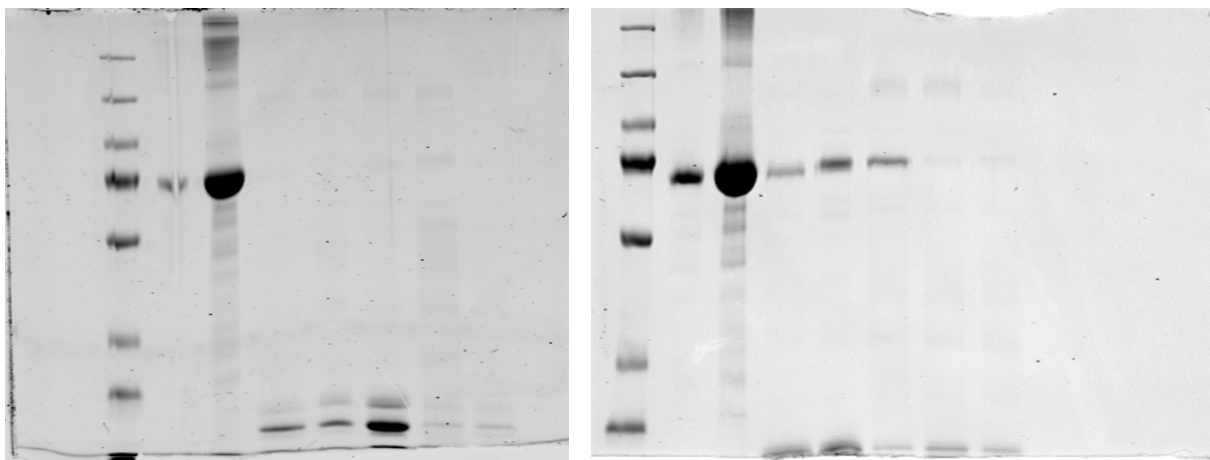
Supplementary Figure S3. Staining of mScarlet-I using an anti-mScarlet-I nanobody. MIP image of a single glomerulus of Pod^{Lifect.mScarlet-I} mouse tissue stained for Lifect.mScarlet-I using an anti mScarlet-I nanobody (cyan) and an anti tdTomato antibody (magenta). Overlap appears in blue. Scale bars – 10 μ m, zoom – 5 μ m.



Supplementary Figure S4. Control iPod^{Lifect.mScarlet-I} mice induced with sesame oil show low number of mScarlet-I positive cells. Representative multiphoton tilesan images of AKS of iPod^{Lifect.mScarlet-I} mouse induced with only sesame oil and no tamoxifen and imaged 5 days later using multiphoton microscopy. **(a), (b)** Tilesan 1 and 2 give an overview of a larger tissue section. Zoom ins (a', a'' and b', b'') highlight glomeruli with (zoom 1) and without (zoom 2) mScarlet-I positive cells. Scale bars – 300 μ m, zoom – 25 μ m.



Supplementary Figure 5. Agarose gel of PCR screening of Lifeact.mScarlet-I founder animals. Gel corresponds to Fig. 1b.



Supplementary Figure 6. Coomassie brilliant blue polyacrylamide gels of spot urine samples from young and old Pod^{Lifeact.mScarlet-I} and Pod^{wt} animals. Gel corresponds to Fig. 2a.

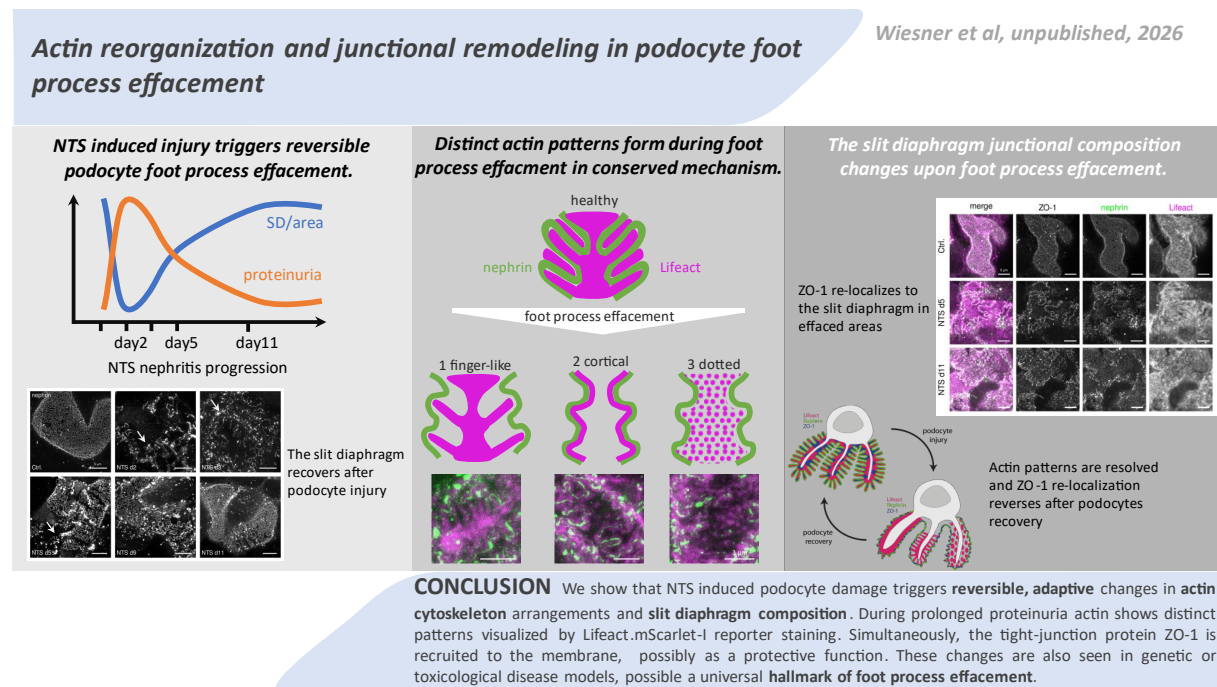
Supplementary Video V1. Intravital microscopy of iPod^{Lifeact.mScarlet-I} mouse glomerulus using multiphoton microscopy. Z-Stack video of glomerulus with induced expression of Lifeact.mScarlet-I (magenta) in podocytes. Blood flow was labeled by i.a. injection of FITC-Dextran. Scale bar – 25 μ m

Supplementary Video V2. 3D projection of iPod^{Lifeact.mScarlet-I} mouse glomerulus using STED microscopy. Z-stack shows nephrin staining (green) and Lifeact.mScarlet-I (magenta) of a single podocyte covering a glomerular capillary. The primary process lacks Lifeact.mScarlet-I signal.

2.3 Actin reorganization and junctional remodeling in podocyte foot processes effacement.

Wiesner Eva, Binz-Lotter Julia, Kroll Marie-Kristin, Bohl Katrin, Lackmann Jan-Wilm, Cabrita Inês, Schermer Bernhard, Benzing Thomas, Hackl Matthias J. - Unpublished manuscript

Graphical Abstract



This manuscript utilized the novel Lifeact.mScarlet-I reporter mouse line to investigate the role of actin in renal disease. One aim was to visualize the actin cytoskeleton and understand which changes are characteristic of podocyte FP effacement. Additionally, we adapted our NTS model to observe later time points to follow disease progression over a longer time period. We were able to portray how the actin cytoskeleton reacts to podocyte injury and describe actin patterns, which develop during the disease course. The slit diaphragm is tightly connected to actin dynamics and therefore, we studied how its junctional composition changes in disease. Alterations in protein localization could hint at mechanistic triggers involved in the development of FPs effacement, posing interesting targets for future studies. We could not only characterize these actin patterns in developing FP effacement, but we also described a novel phenomenon of FPs morphological recovery after injury.

Author contributions

- E.W. performed all experiments, collected and analyzed the data, wrote and revised the manuscript and prepared all manuscript figures, except Suppl. Fig.1D.
- J.B.-L. provided supervision and guidance, designed experiments and revised the manuscript.
- M.K.K prepared LDM glomeruli from FFPE tissue.
- J.-W.L. performed mass spectrometry and data analysis.
- K.B. analyzed proteomic data sets and created Suppl.Fig.1D.
- I.C. revised the manuscript.

B.S. provided funding and guidance.
T.B. provided funding and guidance.
M.J.H. provided supervision, funding and revised the manuscript.

Actin reorganization and junctional remodeling in podocyte foot process effacement.

Abstract

Damage to podocytes results in kidney disease affecting millions of patients worldwide. Actin remodelling is a shared feature in podocyte FP effacement occurring after podocyte injury. However, it remains unclear which processes initiate and orchestrate cytoskeletal changes. Here, we show novel insights into actin dynamics during nephrotoxic serum nephritis and other podocyte disease models. Using Lifeact.mScarlet-I reporter expression and STED microscopy in podocytes, we could show distinct actin patterns occurring during FP effacement, independent of damage origin. Additionally, the protein composition of the slit diaphragm changed in a reversible manner. Tight junction proteins ZO-1 and Claudin-5 appeared at the plasma membrane with the onset of proteinuria. The changes in actin and junctional protein assembly during NTS nephritis were reversible as FP effacement disappeared, proteinuria declined and the podocytes recovered. For the first time, we could show with great spatial and temporal detail how the podocyte actin cytoskeleton adapts to insults and that a reversible response in slit diaphragm dynamics leads to a junctional remodelling better suited for upkeeping filtration barrier function.

Materials & Methods:

List of Antibodies

Antigen	Company & No	Dilution	RRID
Nephrin	R&D Systems, AF4269	1:100	
Nephrin	Fitzgerald, 20R-NP002	1:100	
podocin	Sigma, P0372	1:100	
ZO-1	Invitrogen, 33-9100	1:100	AB_2533147
ZO-2	Invitrogen, 38-9100	1:100	AB_2533390
RFP	Rockland, 600-401-379	1:200	
Claudin-5	Invitrogen, 35-2500	1:100	AB_2533200
α -catenin	Sigma, C2081	1:100	
β -catenin	BD Transduction Labs, 610154	1:100	AB_397555

Mice

Animals were derived and maintained in the CECAD Research Center, University of Cologne, Germany, in individually ventilated cages (Greenline GM500; Tecniplast) at 22°C ($\pm$ 2°C) and a relative humidity of 55% ($\pm$ 5%) under a 12-hour light cycle on sterilized bedding (Aspen wood, Abedd, Germany). Mice had access to a sterilized commercial pelleted diet (Ssniff Spezialdiäten GmbH) and acidified water ad libitum. The microbiological status was examined as recommended by the Federation of European Laboratory Animal Science Associations (FELASA) and mice were free of all listed agents, including opportunists. Mice (C57BL/6N) of both sexes at the age of 4-6 weeks were used. Mice homozygous for Lifeact.mScarlet-I^{fl/fl} were crossed to GCaMP3^{fl/fl} podocin-Cre^{tg/wt} to achieve Lifeact.mScarlet-I/GCaMP3 heterozygosity in all podocytes (Pod^{Lifeact}) [1], [2], [3]. Inducible expression was obtained by using the tamoxifen-responsive podocin-iCreERT2 to generate iPod^{Lifeact} mice [4]. 4-6 week old iPod^{Lifeact} mice received a single Tamoxifen dose of 0.05 mg/g bodyweight via oral gavage. Lifeact.mScarlet-I^{fl/fl} podocin-Cre^{tg/wt} mice were crossed with podocin^{fl/fl} animals to achieve full podocin KO and Lifeact expression in podocytes [5]. Breeding of this mouse line resulted in only one offspring animal of the desired genotype. Due to the severity of the podocin knockout alone, additional Lifeact expression might negatively affect podocytes and further burden renal function, leading to early lethality. Compound heterozygous podocin RQ/AV animals were available in-house as previously published [6]. No animals were excluded; no randomization was performed. Potential

confounders were not controlled and blinding of the study was not performed. N equals the number of mice if not stated otherwise. All experiments were approved by the LAVE NRW, Germany, with the authorization number: 81-02.04.2018.A351 and 81-02.04.2023.A301. Animal experiments complied with LAVE regulations and were conducted in accordance with ARRIVE guidelines.

Induction of nephrotoxic serum nephritis

Mice received a single dose of nephrotoxic serum (Probetex, LOT #612-3T; 8µl/g bodyweight) as an i.p. injection when 4-6 weeks old. Day 1 is regarded as the day of injection. Mice were euthanized with cervical dislocation on the indicated time points (day 2, 3, 5, 9 and 11). The kidneys were removed, decapsulated and further processed for imaging.

Induction of Adriamycin induced FSGS

Due to the young age (4-6 weeks) of mice, Adriamycin was administered intravenously with a small surgical procedure. Before surgery, mice received 0.1 mg/kg BW Buprenorphine. Animals were then anesthetized with 5 % isoflurane and the anesthesia was maintained at 1.5%. After a small incision at the neck, the jugular vein was tied cranially and then cannulated. Adriamycin (or saline for sham surgery) was slowly injected into the vein with a dose of 25 mg/kg bodyweight. After the injection, the vein was ligated and the skin was closed. Animals were screened for the development of proteinuria and euthanized by cervical dislocation 5 days post-surgery. The kidneys were removed, decapsulated and further processed for imaging.

Analysis of proteinuria and serum parameters

For the determination of the albumin-to-creatinine ratio (ACR), both albumin and creatinine concentrations were measured in spot urine samples. Urinary albumin levels were quantified by ELISA. Briefly, 96-well plates were coated with an anti-mouse albumin antibody (1:10.000; A90-134A, Bethyl Laboratories) for 1 h at room temperature. Standards and urine samples were added to the plates and incubated for 1 h. Detection was performed using an HRP-conjugated goat anti-mouse albumin antibody (1:25000; A90-134P, Bethyl Laboratories). Absorbance was measured at 450 nm using a microplate reader (Tecan). Urinary creatinine concentrations were determined using a commercially available colorimetric assay kit (Creatinine (Urinary) Colorimetric Assay Kit, 500701, Cayman Chemical). Serum parameters were determined through the Clinical Chemistry of the University Hospital Cologne and measured on a cobas c systems. Creatinine was determined using CREP2 Creatinine plus ver.2 test (Roche). Urea was determined using UREAL test kit (Roche).

Preparation and imaging of acute kidney slices

Acute slices were prepared according to previously published protocols [7]. Briefly, kidneys were embedded in 4% low-melting agarose and sectioned into 300 µm slices using a vibratome (VT1200 S, Leica). Slices were maintained in carbogenated Krebs–Henseleit buffer (95% O₂/5% CO₂). For imaging, individual slices were transferred to an imaging dish and stabilized with a slice anchor (Warner Instruments) to minimize tissue movement. Confocal imaging was conducted on a STELLARIS 8 STED microscope using an 86× water-immersion objective (HC PL APO 86x/1.2 W motCORR STED W).

STED sample preparation and imaging

Immunofluorescent labeling was performed as previously described [8]. Kidneys were fixed overnight in 4% paraformaldehyde (PFA) and subsequently incubated in a hydrogel solution for 24h. The hydrogel was polymerized for 3h at 37 °C. After washing, the tissue was embedded in 3% agarose and sectioned into 200 µm slices using a vibratome (VT1200 S, Leica). Sections were optically cleared overnight (200 mM boric acid, 4% SDS, pH 8.5) and then incubated with primary antibodies against the target proteins (see ‘List of antibodies’). Confocal and STED imaging of slices was carried out using a Leica TCS SP8 gSTED 3X microscope equipped with a white light laser (WLL) and a 775 nm STED depletion laser (Leica Microsystems).

Image processing

Images were processed using FIJI and Omero.figure web application. slit diaphragm length/area analysis and normalized ZO-1/nephrin ratio were determined using a FIJI macro [6]. Figures were assembled using Adobe Illustrator (Version 29.7.1).

Statistical analysis

For statistical analysis of data, the software Graph Pad Prism (Version 10.6.1. (892) Sep. 8, 2025) was used. We performed an ordinary one-way ANOVA with multiple comparisons or unpaired t-test where necessary. ns – not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Laser dissection microscope

For the isolation of glomeruli from mouse kidney tissue, formalin-fixed paraffin-embedded (FFPE) kidney samples were cut into 5 μm sections and mounted on PEN membrane glass slides (Applied Biosystems). Sections were deparaffinized using a brief descending ethanol series and air-dried overnight. The LMD7 Laser Microdissection Microscope by Leica (DFG-INST 216/1179-1 FUGG) was used to cut glomerular sections from kidney tissue. Isolates were collected in mass-spectrometry buffer (20 ml MS-grade water, 1000 mg SDS, 80 mg Tris). Fifteen glomerular sections were isolated per sample. Glomerular isolates were incubated for 10 minutes at 95 °C followed by Bioruptor sonication for 10 cycles with 30/30-s on/off settings. Samples were incubated with dithiothreitol (10 mM) at 55°C for 30 minutes, followed by the addition of chloroacetamide (40 mM).

Proteomics analysis

Proteomic analysis was conducted by the CECAD Proteomics Facility. Samples were digested using a modified SP3 approach [9], foregoing the second day peptide cleanup and instead loading the complete digest on Evotips (Evosep) after acidification following the vendor protocol. Samples were analyzed on an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific, funded by the Deutsche Forschungsgemeinschaft INST 216/1163-1 FUGG) coupled to an Evosep ONE. The Evosep was run with its Whisper Zoom 20 SPD gradient using an Aurora Elite column (Ionopticks). The mass spectrometer was operated using a WHISH-DIA approach [10]. MS1 spectra were acquired between 400 and 1000 m/z with a resolution of 120k, MS2 spectra were acquired in the same range at 60k resolution in 25 m/z windows, resulting in 24 scans total. Fragments were acquired in a range of 250 to 1500 m/z with a normalized AGC target of 1000% and 30% normalized HCD collision energy. Every 6 scans, an MS1 scan was inserted. Samples were analyzed in Spectronaut 19 (Biognosys) after conversion into htrms format using a canonical murine SwissProt database (UP0589, downloaded 15/01/2025). Afterwards, identified and quantified protein groups were exported and analysis was performed on the iBAQ values. Imputation of missing values was done with R package imputeLCMD using function impute.MinDet with default settings [11]. The resulting data matrix was log transformed for all further analysis. The podocyte damage score (PDS) was calculated on the imputed, log-transformed data matrix using the method “Podocyte Damage Score” and code published in [12] and [13].

Funding

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Acknowledgements

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Introduction

Podocyte health is essential for the maintenance of the glomerular filtration barrier and overall kidney function. Podocyte injury causes filtration failure, ultimately resulting in proteinuria and elevated serum creatinine levels. Podocytes are specialized epithelial cells with an intricate three-dimensional morphology. Primary processes extend from the cell body and further branch into fine protrusions, the foot processes (FPs). While primary processes predominantly consist of microtubules, FPs rely on actin as their main cytoskeletal component. Adjacent, interdigitating FPs are connected by a specialized cell–cell contact known as the slit diaphragm.

The slit diaphragm contains several podocyte-specific proteins, rendering it a specialized junction. Its protein composition is distinct from that of adherens or tight junctions. Proteins like nephrin and podocin localize to the SD and are essential for its structural and functional integrity. Mutations in their corresponding genes cause hereditary nephrotic syndrome, focal segmental glomerular sclerosis (FSGS) and FP effacement [14], [15]. In addition to unique slit diaphragm proteins, it also contains common tight junction proteins, including ZO-1 and Claudin-5. Their loss results in podocyte injury and proteinuria, highlighting their role in filtration barrier maintenance [16], [17], [18].

Podocytes are non-motile, being permanently anchored to the glomerular basement membrane through integrin-based adhesions. Despite their limited motility, podocytes withstand filtration pressure and shear forces, supposedly through an adaptive, contractile actomyosin network [19], [20]. Disruption of actin dynamics is one feature of podocyte injury. Several mutations in actin-associated genes, like *CD2AP* and *ACTN4*, impair cytoskeletal organization and therefore promote FP effacement [21], [22].

The underlying mechanisms of FP effacement have been studied extensively but are not fully understood yet. It is unclear whether alterations in actin organization initiate downstream signaling for cellular adaptation or whether injury-related signaling drives cytoskeletal remodelling. Most structural insights are based on immunogold-labelled protein electron microscopy. Advancements in imaging techniques enable us to visualize cytoskeletal architecture with more detail. We combined our recently published Lifeact.mScarlet-I mouse model, labelling the actin cytoskeleton in great detail, with a nephrotoxic serum (NTS) nephritis model, resulting in transient, reversible FP effacement. By studying disease progression at different time points, we aimed to visualize various stages of actin distribution in a temporal context following damage and repair of podocyte FPs.

Results

The slit diaphragm can recover after podocyte injury.

In order to study actin in FPs effacement, NTS nephritis was induced in 4- to 6-week-old Pod^{Lifeact} mice. This resulted in a transient FP effacement analysed in mice sacrificed on days 2, 3, 5, 9 and 11 after the injection of the serum (Fig.1A). On day 2, the nephrin signal disappeared from the plasma membrane, and FPs were unorganized and effaced (Fig.1B). The nephrin staining for all days showed dots of nephrin in the vicinity of the cell body in circular structures, resembling vesicular internalization (Fig.1B, arrows). At day 2, the NTS model's heterogeneity led to a wide variation between animals regarding their degree of FP effacement. While some animals still presented FP with a morphology close to control animals, others were severely effaced (Suppl.Fig.1A). This interindividual variation was less pronounced on the following time points, suggesting a higher variability in the disease onset.

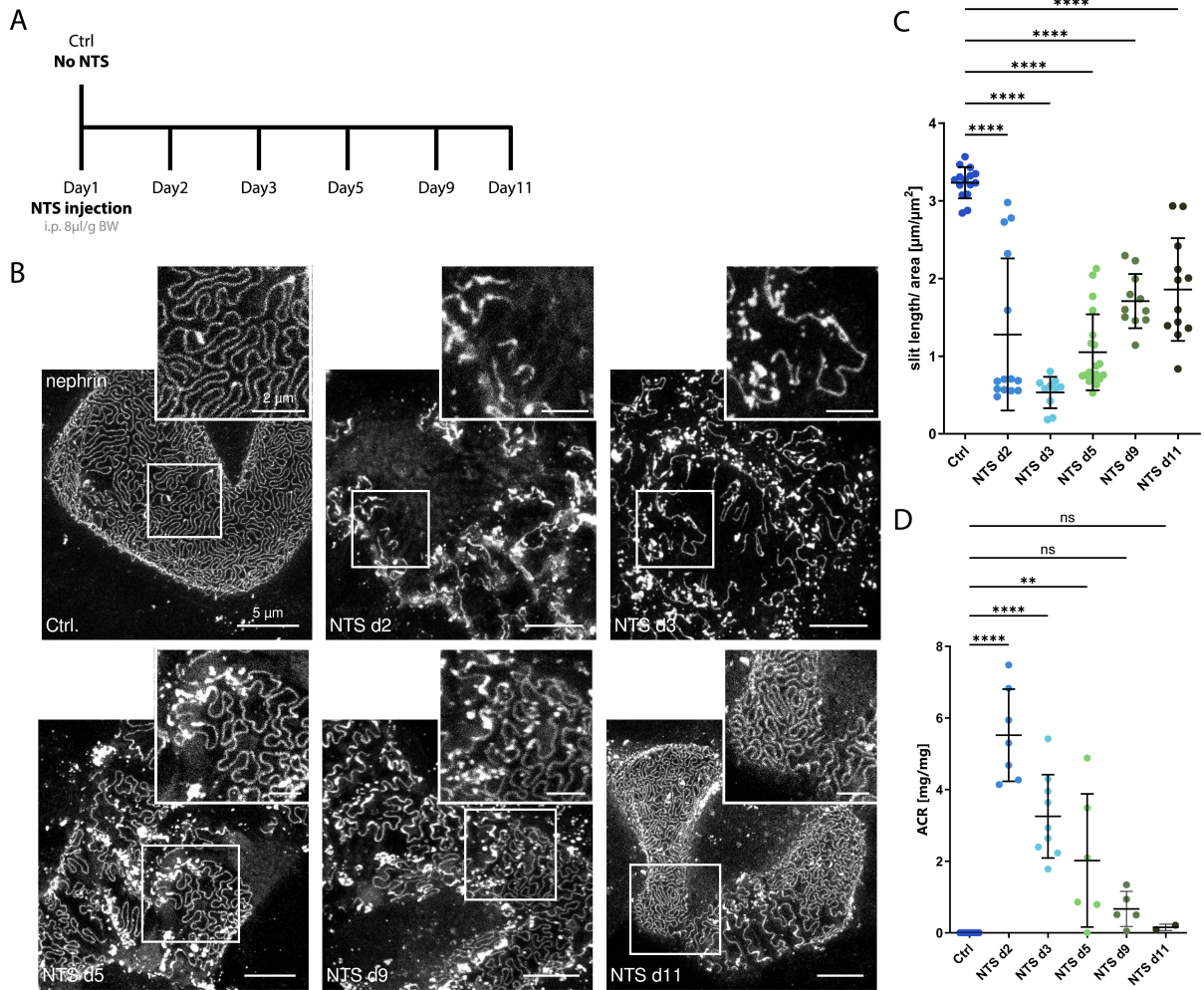


Figure 1: Recovery of the slit diaphragm after NTS induced podocyte injury. A – Mice received a single dose of NTS (8µl/g bodyweight, i.p.). Urine was collected and kidneys were harvested at indicated timepoints after injection. The day of injection was regarded as day1. B – Representative STED images of anti-nephrin staining of glomerular capillaries shows foot process effacement and internalization of nephrin. C – Quantification of slit diaphragm length/area of images as presented in B using a FIJI macro. D – Albumin creatinine ratio (ACR) of animals injected with NTS. Scale bars – 5 µm, zoom – 2 µm. Statistical test: ordinary one-way ANOVA with multiple comparisons; ns – not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. For data points in C: $n=15$ Ctrl, $n=14$ NTS d2, $n=10$ NTS d3, $n=19$ NTS d5, $n=11$ NTS d9, $n=12$ NTS d11; n =glomeruli; For data points in D: $n=11$ Ctrl, $n=7$ NTS d2, $n=9$ NTS d3, $n=6$ NTS d5, $n=5$ NTS d9, $n=2$ NTS d11.

On day 3, the nephrin signal started to increase again, but severe effacement was still present. At day 5 and 9 of nephritis, the slit diaphragm morphology became more regular and elongated, with densely packed FPs and a continuous nephrin staining along the plasma membrane. On day 11, the slit diaphragm pattern recovered, nearly matching control conditions again. FP effacement was still slightly present in some parts of glomeruli, resembling a FSGS-like phenotype. Notably, podocytes' capacity to reorganize FPs was also highlighted by quantification of the slit diaphragm/area (Fig.1C). Analogous to the slit diaphragm/area, clinical parameters like albumin creatinine ratio (ACR), serum creatinine, and urea peaked already at day 2 of NTS nephritis and recovered to physiological levels by day 11 (Fig.1D, Suppl.Fig.1B and C).

To understand the molecular changes in podocyte FP remodelling after NTS injury, we performed proteomic analysis of glomeruli that were dissected using a laser dissection microscope. We applied the novel approach of assigning a podocyte damage score (PDS) to the samples [12]. The PDS showed a heterogeneous onset of disease on day 2 of NTS nephritis, with the most damage occurring on day 5 and a slight improvement to day 9 and 11 (Suppl.Fig.1D).

Distinct patterns in actin cytoskeleton are a hallmark of podocyte damage.

As FP effacement goes hand in hand with ultrastructural changes, we wondered how it affects the actin cytoskeleton. Therefore, we performed immunofluorescence staining for Lifeact and nephrin in Pod^{Lifeact} mice, thus visualizing the actin cytoskeleton and slit diaphragm pattern simultaneously. In healthy podocytes, actin was mostly present in the FPs, covering the entire capillary surface, but not the podocyte cell body (Fig. 2A). Again, on day 3 of NTS nephritis, the slit diaphragm was mostly depleted of nephrin. The FPs were irregularly shaped and large areas were effaced (Fig. 2B). The Lifeact staining revealed an injury-related actin reorganization that can be sub-categorized into three distinct patterns (Fig.2C). The most frequent pattern (1) showed Lifeact distribution near the cell cortex and at the remains of the slit diaphragm, leaving a ‘empty’ cytoplasmic space with no signal. The second pattern consisted of finger-like actin structures branching from a thick actin fibre resembling former healthy FPs (2). However, they were no longer connected to the membrane or outlined by a nephrin signal. The third pattern was mostly found in severely effaced areas and showed a dotted, irregular Lifeact localization (3). All these changes of the actin cytoskeleton were also reversible, as the Lifeact distribution was similar to controls after 9 and 11 days of NTS nephritis (Suppl.Fig.2A). Figure 2D shows a cartoon overview of all three observed patterns.

The endogenous fluorescence of Lifeact.mScarlet-I allowed us to study actin in acute kidney slices (Suppl.Fig.2B). Kidney slices of healthy tamoxifen-inducible iPod^{Lifeact} control animals showed a partial expression of Lifeact.mScarlet-I only in some podocytes and thus enabled a greater contrast in microscopy. Healthy iPod^{Lifeact} presented with normal-shaped FPs. NTS-injured animals (NTS d5) showed similar Lifeact patterns as described above. These experiments in vital tissue verified that the observed patterns were not an artifact of sample fixation and processing.

We then wanted to understand if the observed actin patterns are a distinct feature of NTS nephritis or represent a universal pathological change of actin morphology in different podocyte disease models. We generated mice lacking the full-length podocin protein and express the Lifeact reporter in podocytes (Pod^{Lifeact podocin KO}). The loss of podocin results in a severe, early onset proteinuria with FP effacement at 4 weeks of age (Fig.2E). We found similar actin patterns as in the NTS model, mostly a cortical (1) and dotted actin distribution (3). Another podocyte disease model used was the adriamycin-induced nephropathy as a chemical injury model (Fig.2F). STED microscopy of nephrin-stained tissue revealed areas with a high degree of effacement, while others were not effaced, a typical hallmark of the resulting FSGS. The observed actin morphology was similar to the two previously shown disease models. Severely effaced regions showed a dotted actin distribution (3). We also observed areas that resembled the cortical actin pattern (1). The finger-like arrangement was mostly missing and could not be seen as frequently as in the NTS nephritis model.

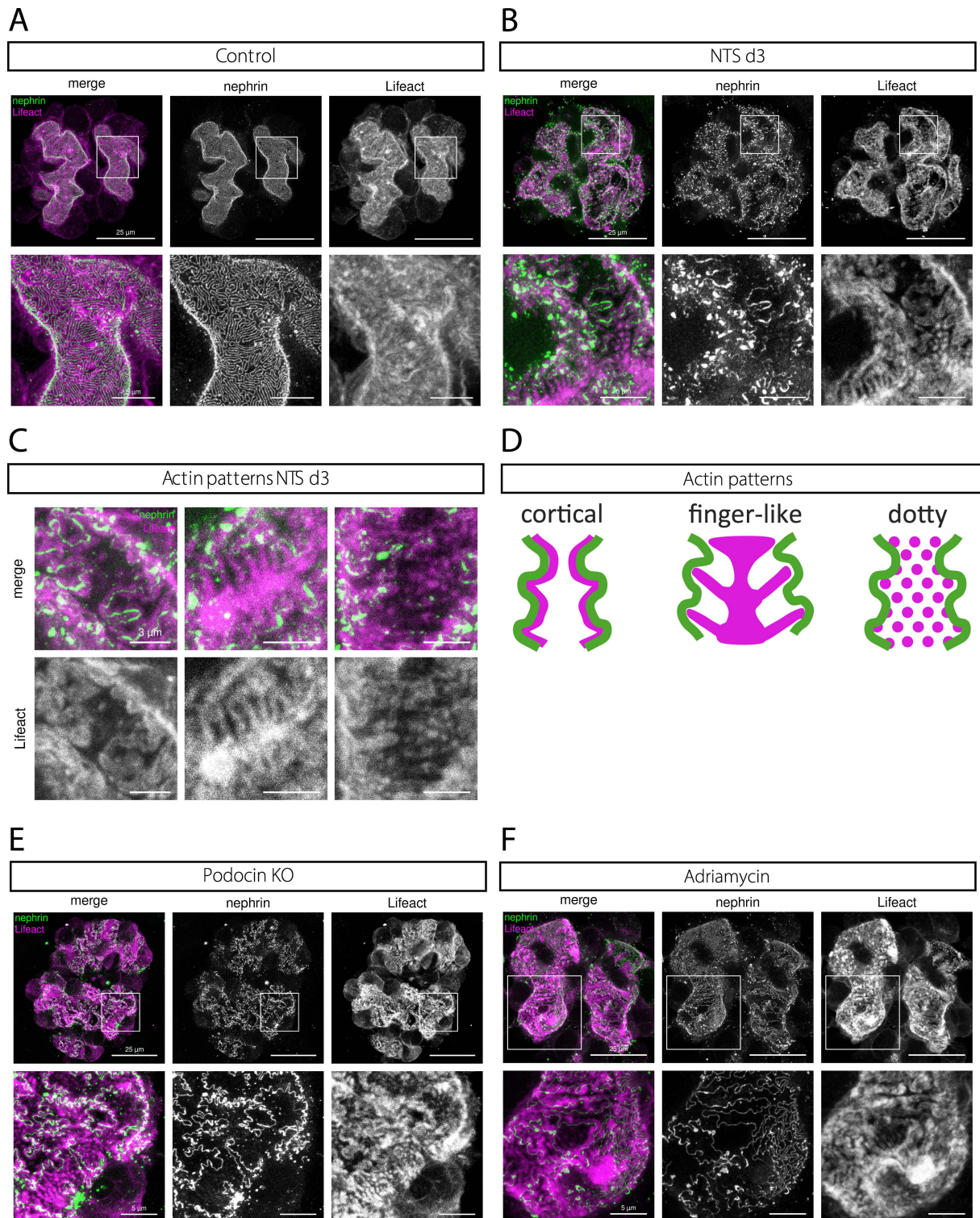


Figure 2: The actin cytoskeleton shows distinct patterns upon podocyte injury. A, B, E, F – Representative maximum intensity projection images of glomerular capillary surface stained for nephrin (green) and Lifeact (magenta). Top rows show overview of whole glomerular capillary. Bottom row zoomed in area. A – Healthy control and B – 3 days after a single dose of NTS. C – Representative images of actin patterns found in NTS injected animals (1) ‘cortical’ Lifeact, (2) ‘finger like’ FP like pattern and (3) a ‘dotted’ Lifeact distribution. D – Images of glomerulus of podocin KO mouse. E – Glomerular capillary of adriamycin injected (25mg/g, i.a.) mouse. Scale bars: A,B,E,F: glomeruli – 25 μm, zoom – 5 μm; C – 3 μm

Actin rearrangements during disease correlate with junctional re-composition.

Our data shows a rapid and dynamic reorganization of the actin cytoskeleton in podocytes during NTS nephritis. As the slit diaphragm is a central point in tethering the podocyte plasma membrane to the actin cytoskeleton, we investigated how the junctional composition of the cell-cell junction was affected during FP effacement.

Tight- and adherens junction proteins such as ZO-1, ZO-2, Claudin-5, α -catenin and β -catenin are known to be present in the slit diaphragm, so we performed immunofluorescent labelling of these proteins to investigate their localization during disease onset. In control animals, ZO-1 showed a ubiquitous distribution throughout the podocyte's FPs (Fig.3A). In injured podocytes, ZO-1 localized mostly to the FP plasma membrane. The other Tjp-family protein, ZO-2, showed only a weak expression in podocytes and its localization was not altered during disease. This was also true for the adherens junction proteins α - and β -catenin. Surprisingly, the tight junction protein Claudin-5 showed a similar re-localization to the slit diaphragm as ZO-1 in injured podocytes.

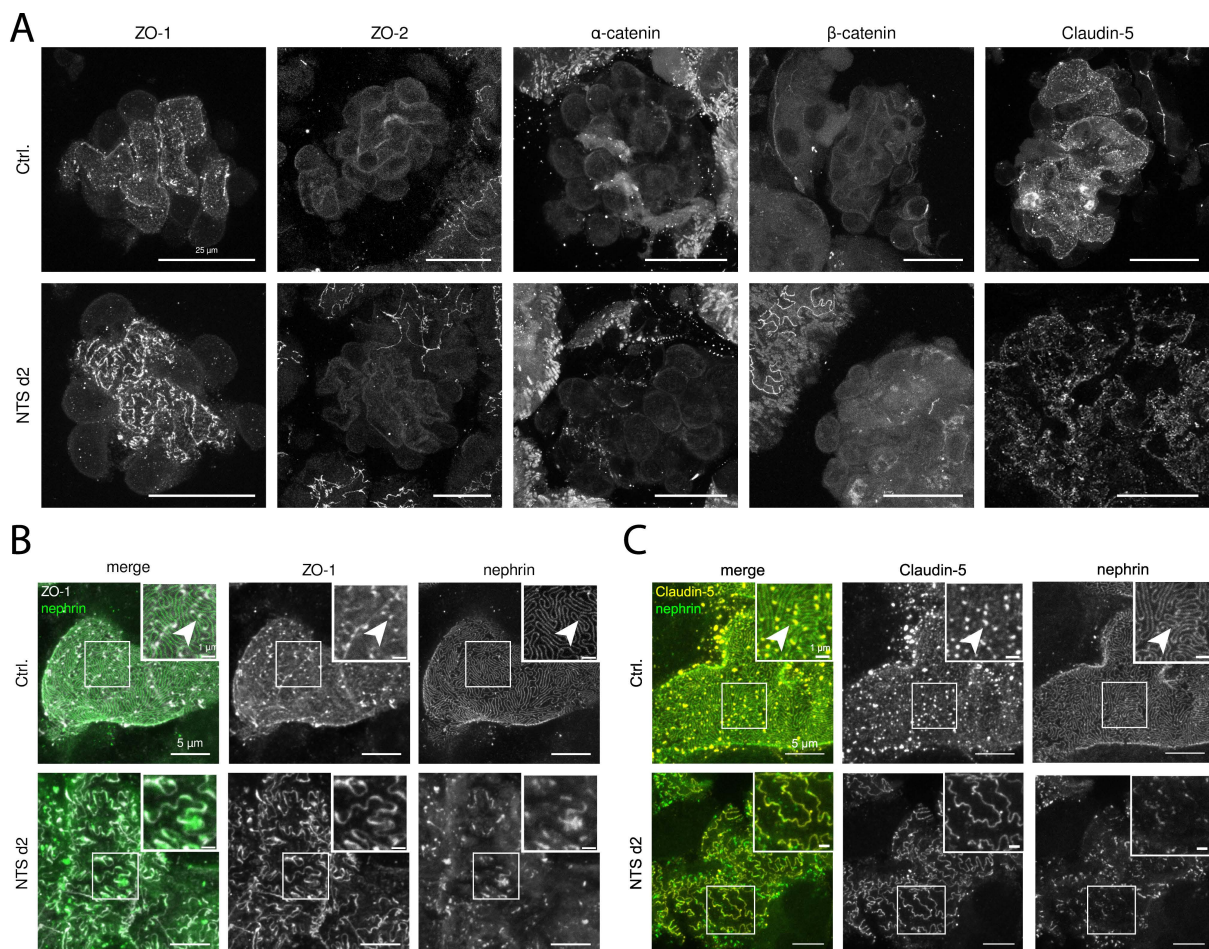


Figure 3: Tight junction proteins re-localize to the SD in FPs effacement. A – Representative images of glomerular capillary surface stained for indicated proteins in healthy control animal (top row) and day 2 of NTS nephritis (bottom). B – Staining for ZO-1 and nephrin in healthy control animal (top row) and day 2 of NTS nephritis (bottom). C – Staining for Claudin-5 and nephrin in healthy control animal (top row) and day 2 of NTS nephritis (bottom). All images are maximum intensity projections. Scale bars: A – 25 μ m, B&C – 5 μ m, zoom – 1 μ m

High-resolution STED microscopy revealed a detailed view of ZO-1 and Claudin-5 localization in relation to the slit diaphragm during effacement. In healthy animals, ZO-1 could be found throughout the FPs but showed the strongest signal at the distal part of the FP, where membrane curvature is at its maximum (Fig.3B zoom, arrowhead). With disease onset at day 2 of NTS, ZO-1

started to specifically localize to the slit diaphragm. Interestingly, Claudin-5 was found at the same unique spot in FPs as ZO-1 in healthy animals. Upon FP effacement, it was found throughout the whole length of the membrane of the cell-cell junction (Fig.3C).

ZO-1 re-localization as a hallmark of podocyte effacement

To further investigate ZO-1 re-localization throughout the NTS nephritis course of injury and recovery, we quantified the signal of ZO-1 in relation to the nephrin signal. We utilized a macro-based detection of fluorescent signals. The ratio of detected ZO-1 and nephrin was then normalized to the amount of nephrin detected, giving the percentage difference. This enabled comparisons between time points and disease models. During the time course of NTS nephritis, the percentage difference peaked on day 2 and remained high on day 3 (Fig.4A). Together with the re-expression of nephrin at the slit diaphragm on day 5, the percentage difference declined and reached levels of control animals at day 9 and 11. Representative images of glomeruli from all days stained for Lifeact, ZO-1 and nephrin are shown in Suppl.Fig.2C.

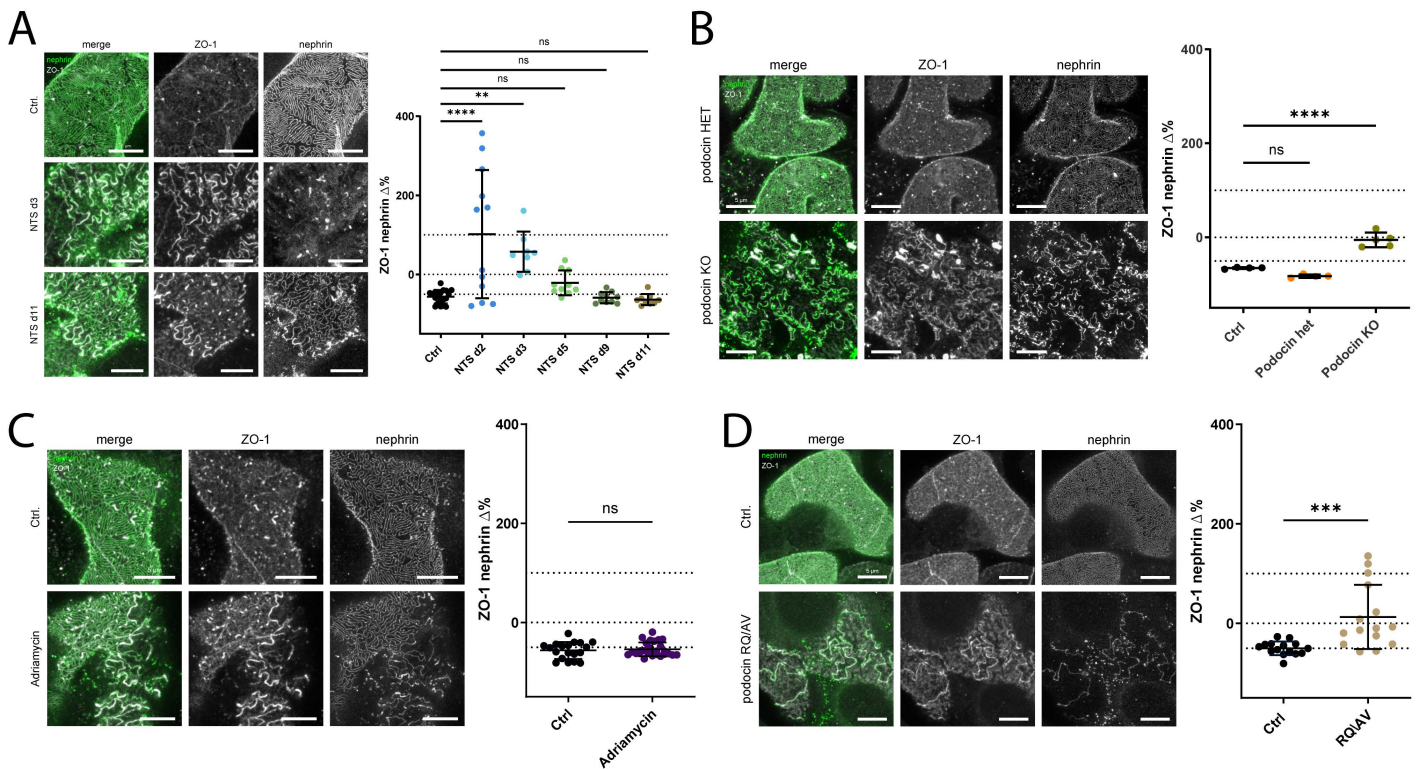


Figure 4: ZO-1 relocates to the slit diaphragm in effaced areas. A-D – Representative maximum intensity projection images of glomerular capillary surface stained for nephrin (green) and ZO-1 (grey). ZO-1 re-localization is quantified by the percentage difference of ZO-1/nephrin signal normalized to the total nephrin signal detected. Images were analyzed using a FIJI macro (s. methods). A – Control and NTS injected animals. Representative images are shown for day 3 and 11 of NTS. B – ZO-1 expression pattern in podocin KO animal (bottom row) and podocin heterozygous control (top row). C – ZO-1 expression pattern in Adriamycin injected (25mg/g, i.a.) mouse (bottom row) and sham surgery control (top row). D – ZO-1 expression pattern in podocin compound heterozygous RQ/AV animal (bottom row) and control (top row). Scale bars – 5μm.

Data points: A – n=19 Ctrl, n=12 NTS d2, n=8 NTS d3, n=10 NTS d5, n=10 NTS d9, n=9 NTS d11 glomeruli; B – n=4 Ctrl, n=4 podocin Het, n=5 podocin KO; C – n=19 Ctrl, n=27 Adriamycin; D – n=15 Ctrl, n=15 RQ/AV. Statistical tests: ordinary one-way ANOVA with multiple comparisons for A+B, unpaired t-test for C+D; ns – not significant, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

For the Pod^{Lifeact/podocin KO} model, a smaller increase in the percentage deviation of ZO-1/nephrin was observed (Fig.4B). Control animals and animals heterozygous for podocin showed a similar ZO-1/nephrin difference as controls. We also quantified the percentage difference of

ZO-1/nephrin in the pharmacological adriamycin disease model (Fig.4C). The average percentage difference did not change compared to sham surgery control animals. In order to exclude the possibility that the occurrence of ZO-1 at the slit diaphragm in effaced areas was influenced by reporter expression, we made use of a genetic injury model: mice carrying a podocin RQ/AV compound heterozygous mutation but no Lifeact reporter. We observed a similar elevation in ZO-1 and nephrin percentage difference in these compound heterozygous animals to the full Pod^{Lifeact podocin KO} (Fig.4D).

Discussion

Our work aimed to gain a better understanding of podocyte FP effacement and its effect on actin cytoskeleton remodelling and slit diaphragm junction composition. The NTS nephritis model showed that podocytes have the capacity to recover from immunological injury. Proteinuria levels peaked on day 2 of NTS nephritis and were accompanied by reduced slit diaphragm length and pathological actin patterns. These changes persisted up to day 5 of nephritis. Starting on this day, a more regular, healthy slit diaphragm morphology was reinstated. On days 9 and 11, podocytes showed a mix of recovered areas and effacement, resembling a FSGS phenotype. Despite severe injury, podocytes could recover and reinstate their unique morphology. It remains unclear whether a further, complete repair of FP morphology occurs in even later timepoints. Glomerular crescents, scarring, and filtration loss are known NTS effects after the initial phase of inflammation [23]. Future experiments could help to delineate whether glomeruli progress into forming crescent or completely recover in the setting of our NTS model.

The involvement of actin deregulation in podocyte injury has been known for a long time [24], [25], [26]. Targeting extracellular proteins through the NTS model led to a disruption of the intracellular actin cytoskeleton, most likely by reduction of adhesion points to membrane proteins, especially the slit diaphragm protein complex [27], [28]. Previous research also described an actin mat upon effacement [26]. The actin patterns we observed were well-conserved between different disease causes. Immunological, chemical, and genetic injury caused podocyte damage, leading to distinct patterns, suggesting their occurrence to be at least partially independent of the type of injury.

The cortical, empty actin pattern and the finger-like pattern seem to be mostly present in areas that are not completely effaced. One could hypothesize that it reinforces the filtration barrier by recruiting proteins to the remains of the slit diaphragm. Additionally, the changed actin localisation could reduce the compression of the FP network on the glomerular basement membrane, which could explain the appearance of the proteinuria peak early during the course of disease (NTS day 2) [6]. The appearance of a subcortical actin network is a typical feature of the calcium-mediated actin reset (CaAR) [29]. The formation of the cortical actin pattern could involve calcium-mediated signaling, but its exact role needs to be studied further. The dotted pattern mostly occurred in areas where individual FPs were completely lost and only one flat podocyte cytoplasm covered the capillary. These dotted structures might be anchored to integrins, helping podocytes to attach more firmly to the basement membrane and withstand increased filtration pressure due to effacement [30].

The downregulation of nephrin following NTS injury is likely to mediate a remodelling of the cell-cell contacts between podocytes. The reoccurrence of tight junction proteins during injury, replacing slit diaphragm proteins, has been demonstrated [18], [31], [32], [33]. Through advancements in microscopy, we were able to examine immunolabeled proteins of the slit diaphragm with high resolution, providing more information than electron microscopy. ZO-1 is a component of the slit diaphragm, interacting with NEPH1, Claudin-5, and actin [16], [34], [35], [36]. During kidney development, it is present in podocytes at the early capillary loop stage [37]. Immunofluorescence images revealed higher staining intensity for ZO-1 than for catenins or ZO-2.

Upon injury, both ZO-1 and Claudin-5 relocate completely to the cell membrane, shifting the junctional composition towards a tight junction. This might be a form of emergency repair mechanism to prevent proteins from passing the filtration barrier [35], [38]. This hypothesis is also supported by the fact that ZO-1 or Claudin-5 knockout in podocytes causes proteinuria and effacement in mice [16], [17]. As those lines were unconditional knock-out lines, it remains unclear if loss during embryonic development or in mature podocytes is responsible for this phenotype. Further experiments with inducible knockout mouse lines of ZO-1 and Claudin-5 are warranted to delineate the role of these proteins after the slit diaphragm has completely developed.

The special localization of Claudin-5 and ZO-1 at the furthest tip of FPs hints at a special function of this area. The slit diaphragm has been suggested to have properties of tricellular junctions, but further validation is needed to confirm involvement of ILDR2 or tricellulin in the re-localization of tight junction proteins in FPs [39].

The reversible nature of the junctional re-composition highlights how important it is that post-mitotic podocytes react with an adaptive response to injury. It remains unclear what triggers the ZO-1 localization change upon podocyte damage. We hypothesized that there must be additional signals, and not only the depletion of nephrin or podocin, for tight junction proteins to reinforce the slit diaphragm cell-cell contact. ZO-1 is very prominently recruited to the slit diaphragm in the podocin KO animal, where nephrin is still present and ZO-1 redistribution also occurred in podocin RQ/AV heterozygous mice.

The correlation of changes in actin patterns and ZO-1 distribution suggests a potential mechanistic link between cytoskeletal properties and the slit diaphragm junction. The actin cytoskeleton is most likely not a direct target of NTS auto-antibodies but was still affected possibly due to changes in molecular tension. ZO-1 conformation has been proposed to function as a molecular tension sensor regulating epithelial homeostasis, depending on the organization of the actin cytoskeleton and myosin-II activity [40]. ZO-1 conformational changes may recruit other proteins to the podocyte membrane, compensating for the loss of podocyte-specific slit diaphragm proteins. The changes might also be triggered by phosphorylation of NEPH1 as a known interactor of ZO-1 or nephrin, with its function as a signaling hub [36], [41], [42], [43], [44].

Our data shows the capacity of podocytes to rebound after damage. However, it was collected for different disease days in different animals. Future work could focus on a longitudinal study design, especially using an abdominal window implanted above the kidney to track changes over time [45].

In conclusion, our data provides novel insights into the reversible nature of FP effacement and how junctional composition and actin dynamics are altered in disease. Podocytes are capable of recovering their slit diaphragm morphology and function upon injury. A universal, adaptive response of the actin cytoskeleton and junctional proteins to FP effacement might provide a future therapeutic target by reinforcing signals for repair and reinstating a functional filtration barrier.

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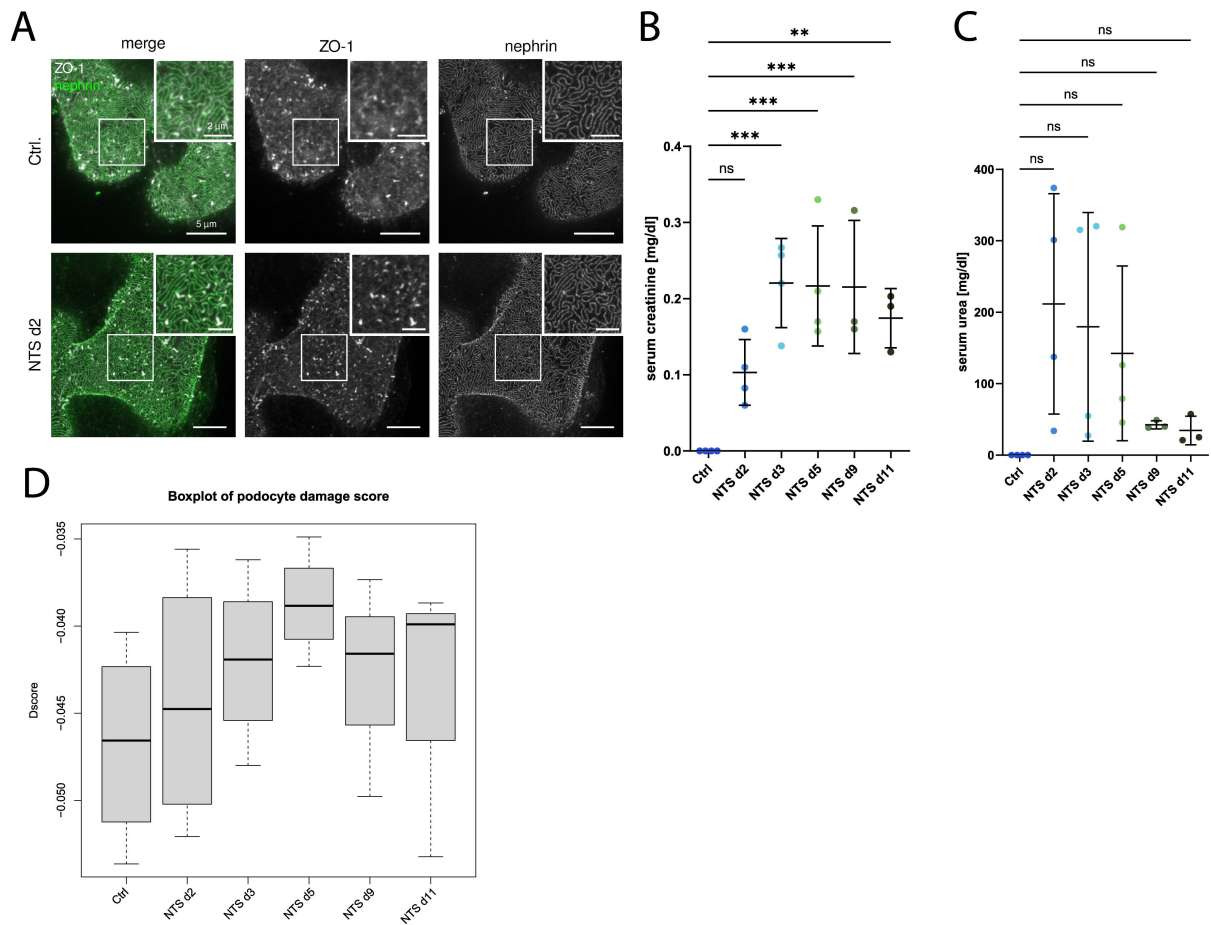
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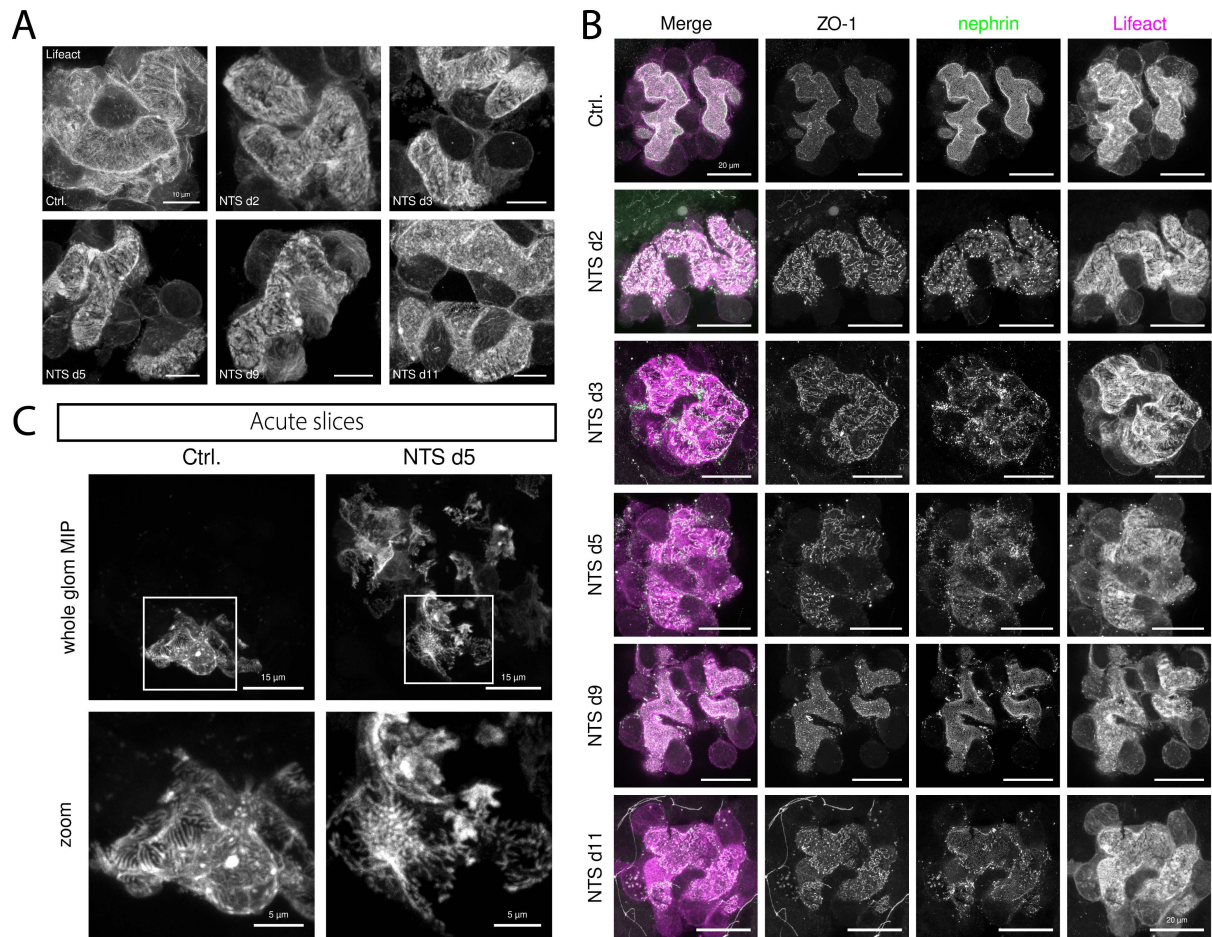
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Supplemental Material



Supplementary Figure 1: Characterization of NTS induced damage. A – Representative maximum intensity projections of nephrin (green) and ZO-1 (white) stained mouse tissue. Upper row shows healthy control animal, bottom row shows tissue of NTS injected mouse on day 2, but not yet proteinuric. Scalebar – 5 μ m. B – Serum creatinine of NTS induced mice. C – Serum urea of NTS induced mice. D – Podocyte damage score for proteomic data of laser dissected glomeruli of NTS injected mice. Data points: B+C – n=4 mice for Ctrl, NTS d2, NTS d3, NTS d5; n=3 mice for NTS d9, NTS d11. D – n=3 for all data points included. Statistical tests: ordinary one-way ANOVA with multiple comparisons for B+C. ns – not significant $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.



Supplementary Figure 2: Lifeact.mScarlet-I staining reveals changes actin distribution during effacement. A – Representative maximum intensity projection images of glomeruli stained for RFP/Lifeact of healthy control and NTS induced mice from day 2 to day 11. Scalebar – 10 μ m. B – Maximum intensity projection images of endogenous Lifeact expression in living podocytes in AKS of iPod^{Lifeact} mice. Left image column shows a healthy control, right column shows NTS d5. Zooms highlight Lifeact.mScarlet-I distribution of single podocytes. Scale bars: glomerulus – 15 μ m, zoom – 5 μ m. C – Representative maximum intensity projection images of glomeruli stained for nephrin (green), ZO-1 (white) and RFP/Lifeact (magenta) of NTS induced mice from day 2 to day 11. Scalebar – 20 μ m.

Discussion

3.1 Calcium signaling and foot process morphology in NTS nephritis in podocytes

3.1.1 Intracellular calcium is dysregulated in podocyte disease

Calcium signaling steers several cellular processes, such as motility, proliferation and specific enzyme activity. Imbalances in calcium homeostasis can ultimately affect cell survival. We saw that baseline calcium concentration was increased in NTS nephritis on day 5 by measuring GCaMP3 fluorescence in glomerular podocytes. We assessed the morphological properties of the slit diaphragm in those glomeruli but found no direct correlation of ultrastructural damage and calcium levels. This could be partially expected as our data evaluated only snapshots of the disease state on a single day after injection of NTS. Our data showed that the amplitude of the increase is uncoupled from the extent of damage. However, the general increase of calcium might still be responsible for initiating damage response mechanisms and regulating effacement dynamics. Previous studies showed calcium is involved in the damage response of podocytes and actin remodeling and therefore FP morphology [54], [98], [99]. In general, elevated but also low calcium levels lead to a harmful imbalance that can cause podocyte damage, rendering correct calcium signaling essential for podocyte health.

A remaining question is how podocytes modulate their response to damage. Generally, damage can trigger a global calcium increase or short calcium transients [89], [100]. It was shown that healthy podocytes do not react with short calcium transients after angiotensin II stimulation *in vivo* but do so after injury with adriamycin [44]. Controversially, data from cell culture models and isolated glomeruli do show that podocytes respond with short calcium peaks after ATP or Angiotensin-II stimulation [101], [102]. However, those model systems lack the unique physiology of podocytes with their intricate cytoskeletal structures and external forces and could be considered more of a podocyte damage model themselves. Also, artificially inducing short calcium transients did not alter podocyte morphology or affect filtration function [103]. It seems likely that short transients, which are quickly reversed, do not lead to cellular injury, but rather that prolonged transients or calcium overload will trigger cellular injury signaling. Recent data also demonstrated that abolishing calcium entry through the mechanosensitive Piezo1 channel can be beneficial if podocytes are already damaged [104]. The data suggests a fine-tuned balance of intracellular calcium concentration is needed to maintain filtration barrier integrity. For future experiments, a long-term observation of intracellular calcium levels for the duration of NTS nephritis development could show changes in baseline calcium and how they affect foot process morphology.

3.1.2 The capacity of foot processes to recover after injury

Additionally, our data shows that FP effacement is a reversible process and the SD recovers partially after NTS nephritis. Proteinuria recedes and SD morphology returns to healthy control levels. In later disease stages, glomerulosclerosis and crescents are described. There is no data on NTS nephritis in mice progressing to end-stage renal disease and kidney failure. This is especially interesting and leads to the question of how NTS-damaged glomeruli fare in persistent disease. There are several possibilities for how glomerular fate progresses. One option is that glomeruli initially recover after the heterologous phase, similar to minimal change disease in children. In later time points in the autologous phase of nephritis, glomeruli might get irreparably damaged by immune cell infiltration and progress to FSGS, which renders them dysfunctional and leads to proteinuria [105]. Another possibility would be that a fraction of glomeruli recover in early

time points while others do not. Since animals show proteinuria in late stages of NTS nephritis, it could be possible that loss of protein occurs due to the FSGS-like glomeruli, which were not able to repair antibody-induced damage.

Our data also demonstrated a plethora of effects of NTS nephritis on podocytes: a calcium increase at baseline, FP effacement and extensive actin reorganization. Podocytes need to adapt with a tailored response to changes in environmental factors, as their loss would be fatal. They seem to possess the potential to adapt fast to altered needs to maintain barrier function as effectively as possible. Losing protein into the urine seems to be less bad than risking a complete detachment from the GBM. Once this capacity to adhere to the basement membrane is exceeded, this might lead to crescent formation and sclerosis as the next level of damage control.

One of the most important questions that remains to be answered is how foot process effacement is initiated. While it has been extensively shown that both calcium signaling and actin dynamics are involved in the process, not much is known which change happens first. Does high calcium lead to rearrangement of actin, or do changes in actin lead to an influx of calcium, possibly by altered cytoskeletal tension? The sequence of events might also be damage-dependent. Proteinuria and changes in actin occur as early as 24h after injection of NTS. The spatio-temporal regulation of both processes could be further studied by investigating NTS-induced damage in even earlier time points and measuring both calcium levels and actin morphology.

One limitation of our study is the lack of data on a possible mechanism behind FP recovery. Altered phospho-signaling, protein binding and changes in tension and forces due to inflammation may lead to FP effacement. The re-composition of the slit diaphragm back to a healthier state could correlate with the clearing of NTS-antibodies from their binding site. A recent study showed that antibody deposition at the FP triggers the formation of antigen-antibody aggregates that are shed as extracellular vesicles into the urine [106]. One could hypothesize that binding of the antibody complex triggers stress response in podocytes, such as elevated calcium and FP effacement, leading to consequent proteinuria. Once the majority of those aggregates are cleared, the podocyte can reinstate proper morphology.

A reporter for the SD could help to study the process of effacement. Utilizing a fluorescently tagged SD protein, it would be possible to follow FP effacement day by day. The implantation of an optical window above the kidney could enable consecutive imaging of SD morphology after disease induction. In general, effacement is a shared occurrence in all podocytopathies. It is worth studying which signals trigger the reconstruction of FPs and what cascade of events occurs to possibly intervene with the development of FP effacement in the first place or trigger healthy restructuring in the second place.

3.2 Actin in foot process effacement

3.2.1 Lifeact.mScarlet-I reveals novel actin dynamics in podocytes

We established Lifeact.mScarlet-I as a novel reporter for actin in podocytes. Historically, the podocyte actin cytoskeleton was visualized by electron microscopy or indirect labelling of actin-binding proteins like synaptopodin. Immunofluorescent labelling of actin filaments usually does not work as fixation and permeabilization damage small FP actin structures and therefore decrease labelling efficiency [107]. Our Lifeact marker showed a bright, cell-specific fluorescence specific to actin. Mice were viable and reproduced normally. Heterozygous expression only mildly affected animals in older age. However, preliminary data from homozygous

animals (not shown) indicate a toxic effect of Lifeact expression on podocytes. Lifeact binding is known to corrupt actin dynamics in various cell types [108], [109]. This toxicity likely depends on Lifeact concentrations and the fluorescent protein tag used. Therefore, an assessment of reporter expression toxicity is crucial to rule out off-target effects simply due to Lifeact binding. Especially in podocytes, it is crucial to avoid interference with actin dynamics as high Lifeact expression might impair contractility and lead to maladaptive response to capillary contraction.

Nevertheless, Lifeact.mScarlet-I is a valuable tool, as we could show three distinct actin patterns appearing during FP effacement in NTS nephritis and other diseases. The three patterns occurred during proteinuria, independent of the origin of podocyte injury. Since FP effacement is a shared feature of both immune-mediated and hereditary podocytopathies, it is not surprising to find podocytes reacting with the same changes in cytoskeletal arrangement for all of them.

The formation of an ‘actin mat’ was already described in 1996 for effaced FPs [34]. More recent studies also showed the formation of sarcomere-like structures, labelled with synaptopodin and myosin 2A [110], [111]. However, our data presents the formation of not only one type of actin structure but three. The different patterns could possibly emerge at different stages of effacement or correlate with the degree of damage (Fig. 7). The adaptation of the actin cytoskeleton to form either of the three patterns could mean they are suited for addressing different needs in stability and motility during specific phases of effacement. One could hypothesize that the finger-like pattern occurs immediately after the retraction of opposing FPs, with the former FPs actin shape intact, while only the membrane recedes. The cortical pattern probably emerges after the actin cytoskeleton has had some time to adapt to membrane retraction and possibly serves as a scaffolding for junctional proteins similar to wound healing processes [112]. The third dotted pattern is most likely what has been formerly described as the ‘actin mat’ occurring in completely effaced areas. The concentration of actin in dots could indicate increased adhesion spots, involving focal adhesion proteins, or could act as additional contractility anchors to adapt to changes in forces upon loss of FPs. So far, most data regarding podocyte actin cytoskeleton dynamics are generated in cell culture and studies greatly lack intravital data. While they might provide an insight into general mechanisms, the different adhesion, lack of 3D orientation and missing environmental factors, such as filtration and blood pressure, render cell culture a suboptimal model for studying podocyte cytoskeleton dynamics.

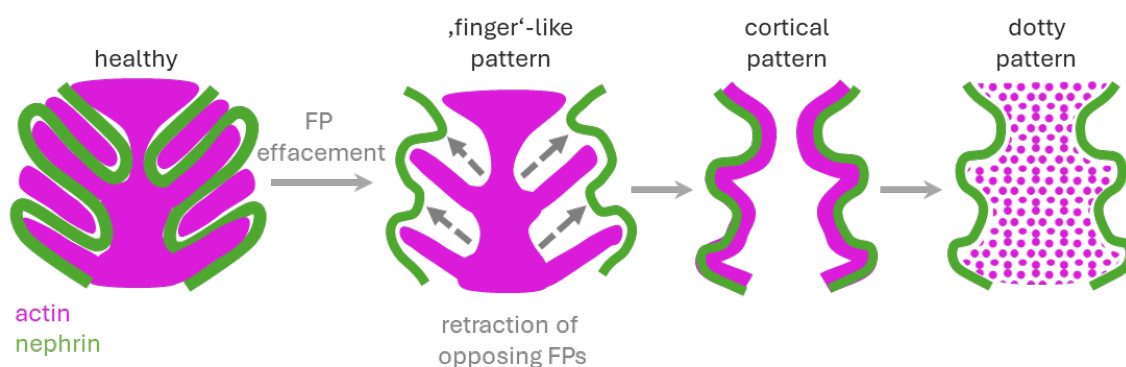


Figure 7: Proposed model for the progression of actin patterns during FP effacement. Actin distribution changes during effacement from a healthy distribution to either of the three patterns: ‘finger’-like, cortical, or dotty.

The observed Lifeact patterns were hard to quantify as their occurrence frequency varies and automated evaluation was not able to distinguish between the three patterns reliably. Another shortcoming is the missing mechanism or signaling pathway driving the formation of those patterns. It remains unclear what type of cytoskeletal, adhesion, or motility proteins are involved

in the formation of the patterns. As the patterns vary in shape, different scaffolding proteins are likely linked to each pattern type. Future work could use Lifeact.mScarlet-I expression to generate a podocyte-specific actin interactome through immunoprecipitation. Tissue-specific expression of Lifeact enabled us to study podocyte actin specifically and independently from other glomerular cell types. In whole glomeruli approaches, like our LDM proteomics data set, podocyte-related changes are often overpowered by effects on the endothelial cells.

Future studies could uncover the details of actin dynamics during foot process effacement and how the adaptation to injury is regulated in podocytes. Possibly elucidating the role of cytoskeletal maintenance and why it is crucial for maintaining filtration barrier integrity.

3.2.2 Podocytopathies of varying disease origin

Here, we presented data for actin remodelling in varying disease models. In general, hereditary models like the knockout of podocin lead to severe impairment in filtration barrier development and early death due to renal failure. The compound heterozygous mutation RQ/AV in the podocin gene mimics a human FSGS phenotype and animals progress more slowly to end-stage kidney disease. With immune-based or toxicological podocyte injury models like NTS or Adriamycin-induced nephropathy, it is possible to initiate disease after the complete development of the filtration slit. All disease models result in proteinuria and FP effacement and share mechanistic pathways of damage to the slit diaphragm, like the observed actin patterns. While podocytes seem to have the capacity to regain healthy morphology after NTS nephritis, this is not the case in hereditary diseases. Continuous damage leads to persistent insult and possibly exhaustion of podocyte adaptability capacity. Surpassing a threshold of podocyte loss ultimately results in glomerulosclerosis. [113] This makes studying NTS nephritis more interesting as it is possible to access signaling mechanisms triggering FP effacement, but also regulatory mechanisms that lead to a reformation of the filtration barrier and therefore could pose as future therapeutic targets.

3.3 Junctional remodeling of the slit diaphragm in foot process effacement

The junctional composition of the slit diaphragm has been investigated since the late '90s. The importance of slit diaphragm proteins for renal health has been undebated, but their specific roles in maintaining filtration barrier function are only becoming clear in recent years.

Our data adds another layer to slit diaphragm composition and how foot process effacement affects reorganization of the junctional complex. We could show the specific localization of adherens and tight junction proteins using immunofluorescence staining with high-resolution STED microscopy. The localization for both ZO-1 and Claudin-5 in healthy controls was not predominantly the whole slit diaphragm but seemed to be specifically the most distal part or 'tip' of the FP. This is contradictory to previous findings that ZO-1 is an integral part of the slit diaphragm [114], [115]. This tip could have a specialized function as it is the most curved part of the slit diaphragm and possibly withstands the greatest tension. Additionally, we found that ZO-1 and Claudin-5 prominently appear at the slit diaphragm during effacement, now localizing along the whole length of the slit diaphragm. This re-localization was reversible, after FPs recovered their morphology from NTS-induced damage. Interestingly, this junctional remodeling was not only a feature of NTS nephritis but also other disease models, such as the RQ/AV compound heterozygous mice, which showed the same patterns, strongly indicating a conserved mechanism in response to effacement. In the FSGS-like phenotype of Adriamycin-injected animals, it became very clear that ZO-1 localization to the slit diaphragm was unique to areas with

effacement, while in parts of glomeruli with conserved morphology, it was only present at the FPs tip. The recruitment of tight junction proteins to the membrane upon injury is most likely a protective effect to reinforce and seal the cell-cell contact. Tight junctions usually prevent the passing of molecules across epithelial cell layers, which would be beneficial for podocytes to prevent proteinuria [41]. Tight junction formation in NTS nephritis has been seen as a feature of early podocyte injury [116], [117]. It is unclear whether a general increase in ZO-1 protein level accounts for the intensified signal at the slit diaphragm or if it is simply a redistribution rather than an expression level change [118].

Specific deletion of ZO-1 or Claudin-5 in podocytes causes proteinuria and effacement [21], [22]. Maybe both ZO-1 and Claudin 5 function as sensing molecules for altered tension, initiating FPs rearrangements. ZO-1 consists of several domains enabling interaction with actin or Neph1, maybe relaying signals to the actin cytoskeleton [119], [120]. In general, the interaction of Neph1 with ZO-1 is important as it was shown to have protective properties in effacement [121], [122]. Additionally, ZO-1 downregulation in the knockout of the focal adhesion protein CHILKBP led to abnormal actin organization, aberrant slit diaphragm protein expression and compromised podocyte filtration capacity [123]. ZO-1 proteins are also involved in linking membrane dynamics to the actin cytoskeleton in NTS nephritis and are known regulators of epithelial organization through actomyosin contraction and membrane traffic [116], [124], [125]. ZO-1 itself was also shown to shuttle into the nucleus of podocytes in culture after puromycin aminonucleoside treatment, possibly regulating damage response pathways and serving as messenger proteins [126].

Our data also indicates that the classical adherens junction proteins, such as vinculin, α - and β -catenin and ZO-2 are not involved in the junctional remodeling during FP effacement, as they did not show a similar pattern to ZO-1 and Claudin-5 re-localization. Studies showed that the knockdown of vinculin and β -catenin or ZO-2 in podocytes did not lead to an overt phenotype but sensitized them to further damage [19], [127], [128].

Similar to the remodeling of the actin cytoskeleton, it remains unclear which signals are needed to trigger junctional recomposition after podocyte damage. Single-cell RNA sequencing might allow us to see changes in pathways related to cell-cell contact formation. However, the changes we observed are likely because of the redistribution of proteins rather than altered gene expression. Slit diaphragm dynamics are tightly regulated through phosphorylation, so perhaps the ZO-1 recruitment occurs due to phosphorylation of Neph1 or other related proteins. Additionally, proximity-based labelling could help to identify novel proteins in the ZO-1/Claudin-5 complex at the tip of FPs and further elucidate its role. It would be interesting to see if the observed actin patterns also occur in ZO-1 podocyte knockout mice, further studying the link between actin cytoskeletal remodelling and junctional recomposition during FP effacement.

Conclusions & Outlook

We aimed to understand how disease affects the calcium – actin axis in glomerular podocytes and characterize pathological changes in calcium homeostasis and actin dynamics. This data provides two main things: we provide new tools to study how podocytes are affected by disease. The first part of this work focused on imaging techniques needed to observe the subtle changes in podocyte morphology. The complex interplay of physiological components, such as filtration pressure and shear stress, combined with multicellular crosstalk in the glomerulus and intricate podocyte biology itself are hard to model and therefore render cell culture and other models insufficient. The discrepancy between in vitro and in vivo data is a major problem in podocyte research, making it essential to study them in their physiological environment. The demand for further improvements of microscopes and intravital techniques is high and future work could aim to resolve the details of the slit diaphragm not in fixed but in living tissue. The Lifeact.mScarlet-I reporter mouse line offers a valuable genetic tool, beneficial for all work studying actin dynamics in intravital and in vivo approaches. The second part of this work utilized the techniques at hand to describe some characteristic changes in podocyte disease. Calcium homeostasis, actin dynamics and junctional remodeling are key factors regulating podocyte survival. Future work should focus on how these three components are regulated and interconnected. Mechanisms explaining how the calcium – actin cytoskeleton axis is regulated are still missing. As most podocytopathies share a dysregulation in calcium or actin dynamics, or both, the potential for finding possible treatment options is enormous. Podocytes numbers in the kidney are few and mostly require targeted approaches to study them. Single-cell technologies are becoming more important and could help to delineate signaling involved in regulating the cellular processes observed in our study.

List of publications

Publications in academic journals

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ. *Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus*. Sci Rep. 2024 Jun 6;14(1):13019. doi: 10.1038/s41598-024-63507-9. PMID: 38844492; PMCID: PMC11156906.

Wiesner E, Binz-Lotter J, Tröder SE, Unnersjö-Jess D, Rutkowski N, Zevnik B, Schermer B, Benzing T, Wedlich-Söldner R, Hackl MJ. *A red fluorescent lifeact marker to study actin morphology in podocytes*. Sci Rep. 2025 Apr 11;15(1):12386. doi: 10.1038/s41598-025-96822-w. PMID: 40216917; PMCID: PMC11992033.

Ester L, **Wiesner E**, Chen H, Ventzke M, Diefenhardt P, Mandel AM, Fabretti F, Brinkkoetter PT, Benzing T, Habbig S, Kann M, Cabrita I, Schermer B. *Transcriptional Regulators YAP and TAZ Have Distinct Abilities to Compensate for One Another in Podocytes*. J Am Soc Nephrol. 2025 Mar 26;36(8):1520-1534. doi: 10.1681/ASN.0000000689. PMID: 40137583; PMCID: PMC12352792.

Rutkowski N, Görlitz F, **Wiesner E**, Binz-Lotter J, Feil S, Feil R, Benzing T, Hackl MJ. *Real-time imaging of cGMP signaling shows pronounced differences between glomerular endothelial cells and podocytes*. Sci Rep. 2024 Oct 30;14(1):26099. doi: 10.1038/s41598-024-76768-1. PMID: 39478086; PMCID: PMC11525973.

Hackl A, Nüsken E, Voggel J, Abo Zed SED, Binz-Lotter J, Unnersjö-Jess D, Müller C, Fink G, Bohl K, **Wiesner E**, Diefenhardt P, Dafinger C, Chen H, Wohlfarth M, Müller RU, Hackl MJ, Schermer B, Nüsken KD, Weber LT. *The effect of mycophenolate mofetil on podocytes in nephrotoxic serum nephritis*. Sci Rep. 2023 Aug 29;13(1):14167. doi: 10.1038/s41598-023-41222-1. PMID: 37644089; PMCID: PMC10465485.

Participation in academic conferences

Oral presentation

15th International Podocyte Conference & 2025 ISGD Meeting, Hamburg, Germany, Early Career Researchers (ECR) Day. *Actin remodeling during foot process effacement affects slit diaphragm junction composition in podocytes*.

Poster Presentations

15th International Podocyte Conference & 2025 ISGD Meeting 2025, Hamburg, Germany

Wiesner E, Binz-Lotter J, Chen H, Schermer B, Wedlich-Söldner R, Benzing T, Hackl MJ. *Actin remodeling during foot process effacement affects slit diaphragm junction composition in podocytes*.

EMBO | EMBL Symposium: Seeing is believing: imaging the molecular processes of life 2023, Heidelberg, Germany

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ. *Combining multiphoton with super-resolution microscopy to investigate podocyte injury in acute murine kidney slices*.

14th International Podocyte Conference 2023, Philadelphia, USA

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ.
Investigating podocyte injury in acute murine kidney slices by combining multiphoton with
super-resolution microscopy.

American Society of Nephrology (ASN) Kidney Week 2022, Orlando, USA

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ.
Combining Multiphoton Microscopy With Super-Resolution Microscopy to Investigate
Podocyte Injury in Acute Murine Kidney Slices.

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Publikationen:

Wiesner E, Binz-Lotter J, Hackl A, Unnersjö-Jess D, Rutkowski N, Benzing T, Hackl MJ. *Correlative multiphoton-STED microscopy of podocyte calcium levels and slit diaphragm ultrastructure in the renal glomerulus*. Sci Rep. 2024 Jun 6;14(1):13019. doi: 10.1038/s41598-024-63507-9. PMID: 38844492; PMCID: PMC11156906.

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Eva Wiesner

Euskirchen, 15.05.2026

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