Intravital multi-photon microscopy reveals several levels of heterogeneity in endocytic uptake by mouse renal proximal tubules

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Abstract

Understanding renal function requires one to integrate the structural complexity of kidney nephrons and the dynamic nature of their cellular processes. Multi-photon fluorescence microscopy is a state-of-the-art imaging technique for *in vivo* analysis of kidney tubules structure and function in real time. This study presents visual evidence for several levels of heterogeneity of proximal tubular endocytic uptake in the superficial renal mouse cortex and illustrates the potential of multi-photon microscopy for providing a comprehensive and dynamic portrayal of renal function.

Most studies address renal function as a whole, thereby ignoring the importance of regional, segmental and cellular differences. Among these, intranephron heterogeneity refers to the well-known structural, biochemical and functional differences between nephron segments [1], and internephron heterogeneity to the two distinct populations of cortical *versus* juxtamedullary nephrons [2]. Regional differences in blood flow and glomerular filtration could also account for functional differences at the level of adjacent, otherwise identical nephrons [3]. Although addressing individual tubules, sophisticated techniques such as micropuncture and microperfusion by necessity ignore their integration at the organ level, including interactions and feed-back from the juxtaglomerular apparatus.

In vivo imaging by single-photon fluorescence confocal microscopy may circumvent these drawbacks, by allowing visualization of renal processes in real time, paving the way to address heterogeneity *in situ* [4]. Furthermore, multi-photon fluorescence microscopy has brought three additional advantages: (*i*) concentration of irradiation to the point of focus; (*ii*) deeper tissue probing thanks to excitation by infrared light and (*iii*) – most importantly – minimized phototoxicity, thus allowing visualization of renal tubules *in vivo* for extended periods of time [5]. During the last 3 years, this technology has yielded major new insights on the dynamics of renal processes in rats [6–10]. In this brief report, we applied multi-photon microscopy in mice and focused on endocytosis: our

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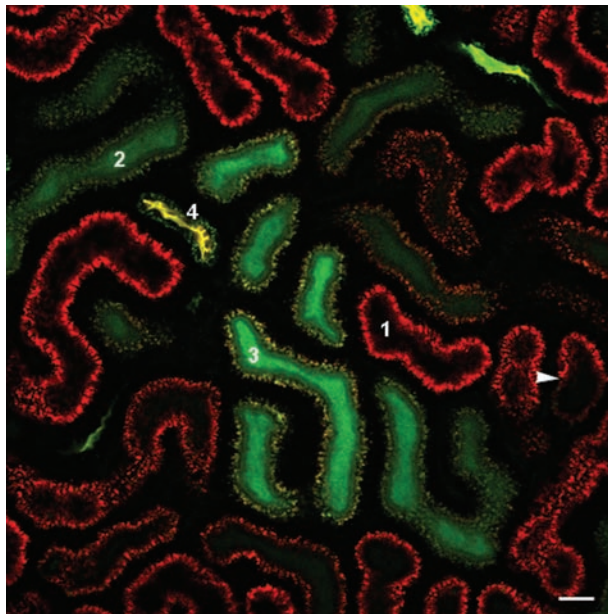E-mail: pierre.courtoy@uclouvain.be

Fig. 1 Overall heterogeneity of the endocytic uptake by cortical proximal tubules of 10 kD dextrans bearing different fluorochromes. This image was taken at 45 min. after the intravenous injection of a mixture of Alexa488- and Alexa568-dextran 10 kD, using a 25x/NA 0.8 water immersion objective. The fluorophores were simultaneously excited at 800 nm, the emitted fluorescence was collected by separate photomultipliers with channels centered at 525 and 600 nm, respectively, and the merged image was generated by superimposition of the two channels. Ultrafiltrated Alexa568-dextran (red signal) has completely disappeared from the lumen and strongly labels the endocytic apparatus of most proximal tubule segments (the identity of this segment was confirmed by autofluorescence before tracer injection [13]). Alexa488-dextran (green) remains in the lumen of more distal segments of proximal tubules, whose endocytic vesicles are labeled by both dextrans (yellow to orange). Opposite changes in intensity between Alexa568- and Alexa488-dextran from profiles **1** (extensive red labelling of the apical endocytic apparatus, no luminal red signal, no detectable green signal) to **4** (yellow lumen, green cellular dots) is indicative of rapid Alexa488-dextran filtration with full uptake in most proximal segments of proximal tubules (**1**), delayed ultrafiltration of Alexa488-dextran with preferential uptake in more distal segments of proximal tubules (**2–3**) and luminal Alexa488-dextran concentration in connecting tubules or initial collecting ducts (**4** still endocytically active). The arrowhead at right indicates a sharp transition within a proximal tubular profile between cells that are either labeled by Alexa568-dextran (upper part) or not (lower part). Scale bar, 20 μm.

analysis reveals a marked heterogeneity for the uptake of fluorescent dextrans in this species and suggests a major effect of the fluorophores.

The experiments were carried out using a Zeiss Axiovert 200M/LSM 510 Meta confocal microscope with a Chameleon infrared laser set at λ^{exc} 800 nm, and performed in accordance with regulations of the National Institute of Health for care and use of laboratory animals. In brief, C57BL male mice, 4–6 months of age, were anaesthetized for several hours under ketamine and xylazine, and the left kidney was exteriorized with minimal surgical procedure. Mice were then placed on the microscope stage in a thermostatted chamber set at 37°C, with the left kidney positioned in a coverslip-bottomed chamber bathed in saline. The renal cortex was best probed at depths between 20 and 60 μm from the capsule. After focusing based on tissue autofluorescence, paired combinations of 10 kD dextrans nominal size conjugated with various fluorophores (15 μg/g body weight of each) were injected into the retro-orbital sinus. In some experiments, Hoechst 33342 (5 μg/g body weight) was added to visualize cell nuclei. All fluorescent tracers (Invitrogen, Eugene, OR, USA) were simultaneously excited and the distinct emitted fluorescent signals were collected by separated photomultipliers.

Heterogeneity between tubular profiles and among adjacent cells of a given profile in C57BL mice kidneys is illustrated in both Figures 1 and 2. Since the analysis was limited to the superficial cortical zone, the marked heterogeneity between tubular profiles for accessibility to, and endocytic labelling by, distinct fluorescent dextrans cannot be due to the differences between cortical and juxtamedullary nephrons, since the latter do not reach the region analysed [11]. Our data therefore primarily reflect intranephron segmental heterogeneity. An abrupt boundary in tracer uptake preference between continuous nephron segments is indeed evidenced in favourable sections (large arrowheads in Fig. 1, right and Fig. 2C, left). Intranephron segmental heterogeneity has already been documented for albumin uptake [12]. As a second level of heterogeneity, clear-cut differences in fluorescent dextran preference between adjacent cells in a random, scattered fashion is also evident in some tubular profiles (Fig. 2C, single *versus* double arrows). On the top of these two documented levels of structural heterogeneity, functional differences in regional blood flow and/or glomerular filtration may add to the complexity of tubular endocytosis, but this level of heterogeneity has not been addressed here. Finally, the strikingly different handling of two different fluorescent dextran
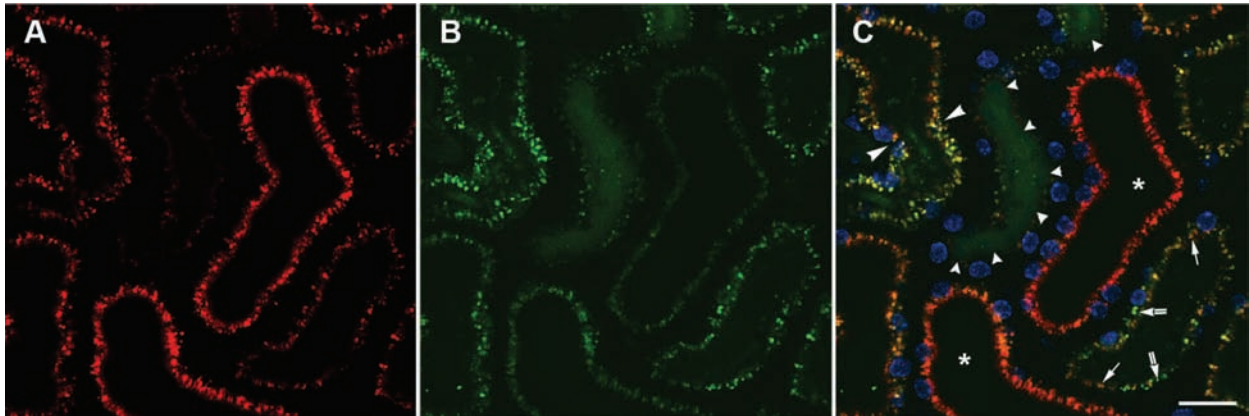
Fig. 2 Fine tissular heterogeneity of fluorescent dextrans uptake by cortical proximal tubules. This image was taken at 60 min. after the intravenous injection of a mixture of Alexa568- (red signal at panel **A**) and fluorescein-dextran 10 kDa (green signal at panel **B**), together with the cell-permeant DNA-intercalating dye, Hoechst 33342, to label the nuclei of renal epithelial cells (blue signal at panel **C**), using a 63×/NA 1.2 water immersion objective. The fluorophores were simultaneously excited at 800 nm, the emitted fluorescence was collected by separate photomultipliers, with channels centered at 600, 525 and 450 nm, respectively, and the multi-color image was generated by superimposition of the three channels (**C**). Different levels of Alexa568- and fluorescein-dextran within endocytic vesicles is evidenced at **C** by a range of colours from red (only Alexa568-dextran) to green (only fluorescein-dextran), with orange to yellow as intermediates. Alexa568-dextran shows rapid ultrafiltration and high uptake in most nephron profiles. Fluorescein dextran shows delayed ultrafiltration (not shown) and preferential uptake by different nephron profiles. The two asterisks indicate proximal tubular profiles with exclusive Alexa568-dextran uptake. Small arrowheads collectively delineate another profile, presumably more distal, still containing fluorescein-dextran in the tubular lumen, and no detectable Alexa568-dextran endocytosis. Paired large arrowheads at the upper left of panel **C** show a sharp boundary between two continuous segments of a proximal tubule with predominant uptake of either Alexa568-dextran (upper part) or fluorescein-dextran (lower part). The lower right part of panel **C** shows heterogeneity of uptake at the single cell level, with scattered preference for Alexa568- (single arrows) or fluorescein-dextran uptake (double arrows). Scale bar, 20 μm.

preparations with presumably superimposable size distribution points to an effect of charge density on ultrafiltration and/or endocytosis efficiency [10].

In conclusion, these are preliminary data which need to be confronted to a thorough biochemical analysis of the size distribution and charge density of tracers used, and call for further studies to better understand the tissular, cellular and molecular basis of the heterogeneity of renal tissular endocytosis. However, it is already clear that multi-photon *in vivo* imaging is a proper tool to achieve this goal. Moreover, this approach opens fascinating perspectives for detailed investigations of the renal function in transgenic mouse models.

Acknowledgements

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