Applications of Two-Photon Microscopy

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Fluorescence microscopy has represented a crucial technique to explore the cellular and molecular mechanisms in the field of biomedicine. However, the conventional one-photon microscopy exhibits many limitations when living samples are imaged. The new technologies, including two-photon microscopy (2PM), have considerably improved the in vivo study of pathophysiological processes, allowing the investigators to overcome the limits displayed by previous techniques. 2PM enables the real-time intravital imaging of the biological functions in different organs at cellular and subcellular resolution thanks to its improved laser penetration and less phototoxicity. The development of more sensitive detectors and long-wavelength fluorescent dyes as well as the implementation of semi-automatic software for data analysis allowed to gain insights in essential physiological functions, expanding the frontiers of cellular and molecular imaging. The future applications of 2PM are promising to push the intravital microscopy beyond the existing limits.

intravital imaging two-photon microscopy kidney disease

1. Renal Autofluorescence to Study Metabolic Functions

The renal metabolic functions may be studied indirectly by easily analyzing the urine samples collected as easily accessible liquid biopsy from individuals. The specific urinary patterns of molecules detected is an indirect measure of the pathophysiological status of patients [1][2][3].

Instead, the application of 2PM provides unique and direct opportunities to perform morphological and functional studies, even without external labeling or exogenous dyes, taking advantage of the endogenous autofluorescence properties displayed by several molecules. For example, the lysosomal and mitochondrial NADH fluorescence naturally exhibited by the renal tubules enables the visualization of some kidney structures [4]. This intense autofluorescence allows to recognize the proximal tubules that are very rich in mitochondria and lysosomes, while other nephron segments poor in such organelles, such as the distal tubules, and the collecting ducts appear as dark empty patches [5]. The superficial glomeruli, lacking of any fluorescence, appear likewise as large dark empty spaces close to proximal tubules, as visibly appreciable in Munich Wistar Frömter (MWF) rats. Thus, the autofluorescence can be very supportive in recognizing and defining the renal components without using exogenous dyes.

This approach has been important to clarify the metabolic profile in S1 and S2 proximal tubules at the physiological level. Indeed, Bugarski et al. demonstrated that the mitochondrial NAD(P)H expression is comparable between the two populations of tubules. However, S2 proximal tubules displayed a more intense signal at the cytosolic level and

this seems to be justified by their main function to maintain the pool of cellular glutathione. Moreover, a higher expression of flavoproteins is also displayed in S2 than S1, reflecting a different regulation of glutamine and nitrogen metabolism [6][7]. In addition, it was shown that S2 proximal tubules are more dependent on glucose metabolism than S1 tubules as confirmed by the drastic signal reduction in this nephron segment upon the stimulation with a glucose inhibitor [6]. This is in agreement with previous studies showing a damage of rat S2 isolated tubules when a glucose inhibitor was used [8].

As shown by Hall et al., NADH also provides a crucial read out of the metabolic state of renal tubules after renal injury, such as ischemia reperfusion injury (IRI). In particular, researchers demonstrated that following IRI, the membrane potential quickly dissipates leading in turn to the fragmentation and shortening of the mitochondria in proximal tubules, while the distal tubules better maintained the membrane potential [9]. This may be crucial to investigate the electron transport chain function, which provides information regarding the redox state of the mitochondria [10]. These data pave the way toward the therapeutic application of this technical approach to monitor the eventual improvement of the membrane potential and the overall metabolic function, following the administration of IRI-targeting drugs.

Notwithstanding, the main drawback in using the renal autofluorescence to investigate the metabolic functions is evidenced when external probes are used concurrently. In fact, in these cases, the tissue autofluorescence may overlap with the emission signals of the external probes, rendering the data obtained from the analysis doubtful [11].

2. Second Harmonic Generation (SHG) to Study Renal Fibrosis

Additional applications of 2PM make use of the second harmonic generation (SHG) technology, often called frequency doubling, which is a non-linear optic phenomenon that takes advantage not of absorption but of the Rayleigh scattering. In particular, two photons with the same frequency interact with a non-linear material and are finally ‘combined’ resulting in frequency-doubled photons [12][13][14]. Consequently, SHG signals are revealed at the half of excitation wavelength, and can be detected simultaneously with the renal autofluorescence, providing the overall imaging perspective of the organ. The advantages obtained by this imaging approach are understandable. SHG is particularly beneficial as it does not require external fluorescence labeling, hence circumventing the effects of photobleaching and the issues related to molecules administration.

One main application of SHG relies on the possibility to visualize collagen fibers [15], as well as muscles myosin [16] and microtubules [17], showing high specificity when the signal is detected. Indeed, SHG allows to distinguish type I and type III collagen fibers, whereas non-fibrillar material, such as type IV collagen, cannot be revealed in tissues. Additionally, no tissue preparation is required: fresh, fixed, and frozen preparations as well as living organs can be analyzed, ensuring the reproducibility of the technique and minimizing the variability shown by conventional histological techniques. Furthermore, 3D reconstruction of imaged SHG is possible in living samples thanks to the high penetration power of the multiphoton laser. In turn, this permits to perform volumetric analysis of the tissue of interest. Accordingly, the application of SHG in the field of nephrology is well documented and many research

groups have used SHG to image and quantify the fibrosis progression in renal diseases, showing the high potentiality as a pejorative predictor of nephropathies in many renal injuries [15][18][19] and in translational cancer research [20].

3. Single-Nephron Glomerular Filtration Rate (SNGFR) Assessment

A reliable measure of kidney function is provided by the glomerular filtration rate (GFR), which is one of the most important renal parameters taken into account by clinicians for diagnosing renal disorders or monitoring chronic renal diseases [21]. The kidney functionality with the measurement of the GFR, as well as the kidney volume and the renal clearance may be complemented by assessment with other imaging techniques such as dynamic micro-PET (Positron Emission Tomography) analysis after injection of radiotracers [22].

The GFR indicates the overall filtration function of the kidney, whereas the single-nephron GFR (SNGFR) considers the individual filtration events, assessing the function of individual nephrons. Since its discovery, the evaluation of SNGFR has been established as one of the key parameters to evaluate the renal function [23]. In fact, in some pathological conditions, such as the diabetic nephropathy, the total GFR may remain unchanged, even after a significative nephron loss (50%), because of the compensatory hyperfiltration of single nephrons [23]. For these reasons, evaluating the SNGFR provides more accurate and precise information regarding the glomerulus dynamics, as well as the determination of the mechanisms of tubular reabsorption and secretion.

Historically, the micropuncture was the first technique employed to elucidate the mechanisms of renal function and has been extensively used for many years as the gold standard technique for the SNGFR calculation in vivo. However, this technique is laborious and demanding in virtue of the very complex surgical preparation of the animals and the sophisticated equipment required [24]. For these reasons, the assessment of SNGFR represents one of the most attractive applications of 2PM in kidney physiology. Accordingly, Kang et al. developed and implemented a 2PM-based method, consisting in the observation and quantification of the fluorescent decay time of a low-molecular-weight dye between two regions of interest selected within a tubule [25]. In particular, they used multiphoton resonant scanners, which are optional galvanometric mirrors able to perform higher full-frame acquisitions (>20 Hz) than basic 2PM. This approach provided reliable data comparable to the old-fashioned micropuncture. Notwithstanding, the implementation of 2PM with resonant scanners requires additional costs for the users. Furthermore, the high-speed acquisition offered by this imaging modality may increase the noise of the detected signal, hence forcing the investigators to use imaging software to analyze the data and improve their quality.

Instead, an innovative method that does not employ the resonant scanners was recently used to assess the SNGFR through the application of the linescan tool [26]. Linescan is a well-established 2PM method mainly exploited in kidney physiology to assess the erythrocytes velocity in renal vessels [27]. Linescan permits to acquire a region of interest (ROI) repetitive times reaching a very high temporal resolution, since the acquisition is focused only on a drawn line, instead of the entire field of view. This novel application, therefore, does not require the

implementation of expensive resonant scanners and can obtain even faster measurements (>400 Hz) than previous multiphoton approaches. Standing as a faster method, it permits to measure a higher number of tubules in the same animal during the experiment. This, in turn, would allow the investigation of new complex biological questions, such as the nephron heterogeneity. The linescan tool has been validated in models of increased (i.e., following low-dose dopamine administration) and reduced (i.e., following IRI) SNGFR, showing results comparable with conventional micropuncture and previous full-frame acquisition [26]. Notably, while conventional micropuncture only allows to measure a few tubules per single animal for SNGFR experiments, the linescan tool allows to analyze on average 15 tubules, hence improving the reliability of the study. The novel linescan-based approach is a reliable tool for the in vivo assessment of SNGFR in health and disease, thus representing a promising method for future preclinical investigations.

4. Organic Cations Transport Evaluation

Further applications of 2PM technology include the evaluation of the organic cations (OC) transport throughout the kidney. The excretion of OC mainly occurs along the proximal tubules by a secretion mechanism [28]. For the regulation of such mechanisms in humans, the organic cation transporter 2 (OCT2) is involved in the basolateral uptake of many OC in tubular epithelial cells, whereas the multidrug and toxin extrusion 1 and 2 proteins (MATE1, MATE2-K) mediate the apical secretion of OC. In rodents, the tubular secretion is mediated by the basolateral OCT1 and OCT2 transporters, and by the apical MATE1 [29]. Because of this fine regulation, the toxic substances (including antibiotics and diuretics) and the endogenous metabolites (such as catecholamines) are efficiently removed from the blood. Among these molecules, the uremic toxins are particularly dangerous since their accumulation can lead to chronic kidney disease [30]. Therefore, the development of methods to in-depth investigate the OC transport is required for the formulation of drugs and optimization of dosages.

The fluorescent organic cation 4-(4-(dimethylamino)styryl)-N-methylpyridinium (Asp+) has been proposed as a valid dye to study the OC transport in the kidney. The work of Hörbelt et al. [31] demonstrated for the first time that the secretion of OC can be elucidated in vivo in the rat kidney by means of the 2PM and Asp+ marker. Accordingly, researchers monitored the continuous distribution of the fluorescent signal in the renal tubules upon the endovenous administration of Asp+.

A very recent study exploited the potential of 2PM to extend the knowledge of renal OC transport by using the Asp+ dye [32]. The first attempt of researchers was to reproduce the results obtained by Hörbelt. The presence of the signal in the tubular lumen and the strong fluorescence along the apical membrane of proximal tubules detected during the in vivo experiments induced the investigators to test the hypothesis of a binding between Asp+ and albumin. Therefore, a combination of in vivo 2PM and PET approaches was used to investigate the binding of ASP+ with serum albumin. The data obtained by this work clarified that Asp+ may not be a convenient probe for the in vivo evaluation of OC transport, since the detected signal is likely affected by the presence of albumin. These in vivo observations have been demonstrated through in vitro assays, showing an enhancement of the Asp+ signal in presence of bovine serum albumin (BSA) and a blue shift of the emission peak. Finally, further

confirmation comes by considering that the high Asp+ signal was detected in old rats, which likely show high levels of albumin in the ultrafiltrate, while this marker was not detectable in the lumen of young rats [31].

5. Renal Tissue Regeneration

Mammalian cells have limited lifespan; therefore, they need to be continuously replaced to ensure the morphological and functional integrity of an organism. Compared to other organs, such as liver and skin, whose cells are constantly renewed, renal tissue holds a lower cell turnover, thus showing limited regenerative ability. However, the replenishment of renal cells is necessary to preserve the kidney function, despite the mechanisms of tissue regeneration still not being clear [33].

The conventional experimental approaches are not able to follow the renal regenerative process over the time, leaving a gap of knowledge in this field. The advent of 2PM has allowed to constantly monitor in real-time the dynamic reparative process in the kidney with unprecedent results. In particular, Schiessl et al. demonstrated with 2PM analysis, after applying an abdominal window on the mouse, that the interstitial cells are involved in the regeneration of the renal epithelium by a PDGFβ-mediated process [34].

Along the same lines, Zhang and colleagues recently exploited the 2PM and the abdominal window to investigate the regenerative therapies following acute kidney injury (AKI) [35]. In detail, researchers tested the ability of mesenchymal stem cells (MSC)-derived microvesicles (MVs) to regenerate the renal tissue in a transgenic mouse model of AKI. The data obtained showed that the injected MVs were able to travel to the injured kidney and promote the formation of efficient renal tubules through a Sox9-mediated mechanism. The therapeutic effect of MSC-derived MVs was further demonstrated by the evaluation of renal function. Indeed, the progression of renal fibrosis as well as the increase of blood urea nitrogen and serum creatinine typically detected after AKI were significantly reduced following the MVs administration [35]. These results show that 2PM and the abdominal window constitute a powerful combination to track in real time the distribution of single MVs in renal tissue and the repair process after a renal injury. This approach is promising for future studies aiming to explore the regeneration of other abdominal organs.

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