Label-free fluorescence lifetime and second harmonic generation imaging microscopy improves quantification of experimental renal fibrosis

see commentary on page 941

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All forms of progressive renal diseases develop a final pathway of tubulointerstitial fibrosis and glomerulosclerosis. Renal fibrosis is usually quantified using histological staining, a process that is time-consuming and pathologist dependent. Here we develop a fast and operator-independent method to measure fibrosis utilizing the murine unilateral ureteral obstruction model which manifests a time-dependent fibrotic increase in obstructed kidneys while the contralateral kidneys are used as controls. After ureteral obstruction, kidneys were analyzed at 7, 14, and 21 days. Fibrosis was quantified using fluorescence lifetime imaging (FLIM) and second harmonic generation (SHG) in a Deep Imaging via Enhanced photon Recovery deep tissue imaging microscope. This microscope was developed for deep tissue along with second and third harmonic generation imaging and has extraordinary sensitivity toward harmonic generation. SHG data suggest the presence of more fibrillar collagen in the obstructed kidneys. The combination of short-wavelength FLIM and SHG analysis results in a robust assessment procedure independent of observer interpretation and let us create criteria to quantify the extent of fibrosis directly from the image. Thus, the FLIM-SHG technique shows remarkable improvement in quantification of renal fibrosis compared to standard histological techniques.

Kidney International (2016) **90,** 1123–1128; http://dx.doi.org/10.1016/j.kint.2016.06.030

KEYWORDS: autofluorescence; collagen; fibrosis; FLIM; SHG; UUO

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Received 7 April 2016; revised 7 June 2016; accepted 23 June 2016; published online 21 August 2016

Pathologically, chronic kidney disease is characterized by replacement of normal kidney tissues with extracellular matrix components, including fibrillar collagen (types I and III collagen).[1] Given the importance of fibrosis in the progression of chronic kidney disease, many preclinical studies in kidney disease use fibrosis as an endpoint. Quantification of fibrosis is performed using both biochemical and histological techniques.[2] These techniques include hydroxyproline measurement, Masson's trichrome stain, picrosirius red stain, and immunohistochemistry (IHC) for collagen I and III. The histologic studies are limited by batch-to-batch variation in staining as well as inter- and intra-observer variability when visual assessment is used to quantify collagen.[3,4] Recent data have shown automated analysis of polarized picrosirius red images provides accurate and reproducible collagen measurements; however, there is still no "gold-standard" histologic technique to measure kidney fibrosis that performs ideally in all situations.[4,5]

Second harmonic generation (SHG) and fluorescence imaging have been widely used to measure fibrosis in tissues.[6–9] Fibrillar collagens have a noncentrosymmetric structure and thus can give rise to SHG.[6] Previous studies have mainly used the same objective for both excitation and collection of SHG signal (backward generation), which is less sensitive than forward generation.[6–7] The previously described Deep Imaging via Enhanced-photon Recovery (DIVER) system uses forward detection and exhibits high sensitivity to SHG.[8,10–12]

Fluorescence imaging of fibrotic tissues typically involves extrinsic fluorescent labeling and autofluorescence imaging with a 2-photon excitation scheme.[13–14] However, fluorescence lifetime imaging (FLIM) for either labeled samples or tissue autofluorescence has never been employed for characterizing fibrosis in kidneys. A phasor approach to FLIM imaging along with SHG generation can quantify fibrosis.[15–18] The signal originating from SHG and short wavelength fluorescence can be separated based on the lifetime of a signal.[19] Fluorescence is a delayed emission and thus has a nonzero lifetime. SHG is a coherent emission, the signal from which resembles the laser pulse and thus has a lifetime of zero. A major benefit of combined SHG–FLIM imaging is to simplify the sample preparation and create a ratiometric

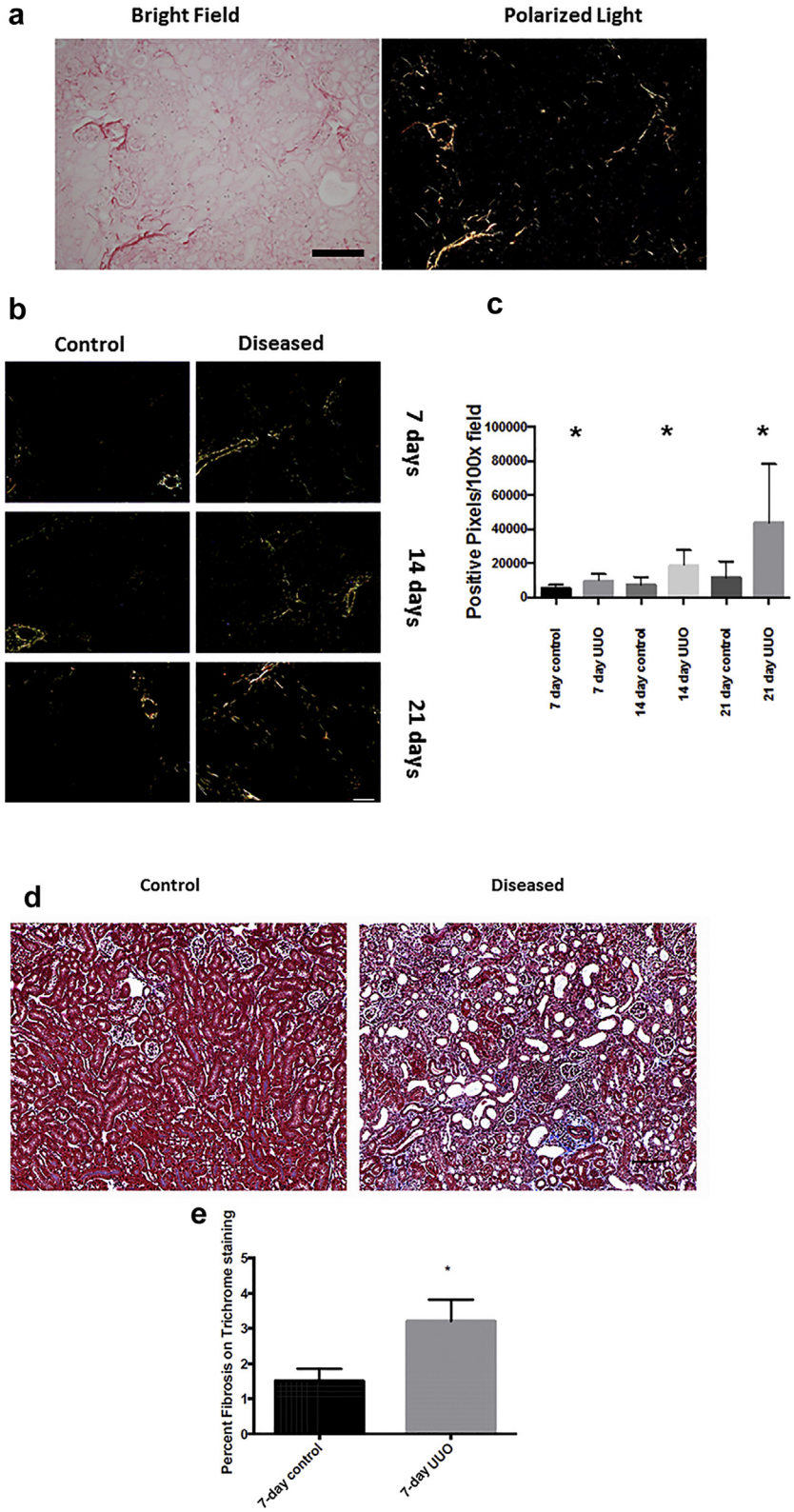

Figure 1 | Quantification of fibrosis using histologic methods. (**a–c**) Picrosirius red–stained kidneys were visualized under both bright field microscopy and polarized light microscopy. (**a**) Representative original magnification ×100 images of the same section visualized with both techniques. (**b,c; asterisk**) There is a time-dependent increase in collagen deposition seen using polarized light microscopy. *$P < 0.05$ versus control at same time point. (**d**) Masson's trichrome staining was also performed and revealed tubular damage and collagen deposition. Original magnification ×100 representative images are shown. Seven days after UUO, there are a significant number of dilated tubules and collagen fibrils. (**e**) Quantification of collagen shows a significant increase 7 days after UUO. *$P < 0.05$ versus control. Bar = 25 μm. UUO, unilateral ureteral obstruction.