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Characterizing Fibrosis in Mouse Kidney using Label Free Fluorescence Lifetime and Second Harmonic Generation Imaging Microscopy in Unilateral Ureteral Obstruction Model

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Abstract

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. The work described here shows the development of a fast and operator-independent method to measure fibrosis. To study renal fibrosis, the unilateral ureteral obstruction (UUO) model was chosen. Mice develop a time-dependent increase in obstructed kidneys; contralateral kidneys are used as controls. After UUO, kidneys were analyzed at three time points: 7 days, 14 days, and 21 days. Fibrosis was investigated using FLIM (Fluorescence Lifetime Imaging) and SHG (Second Harmonic Generation) in the deep tissue imaging microscope called DIVER (Deep Imaging via Enhanced photon Recovery). This microscope was developed for deep tissue and SHG and THG (Third Harmonic Generation) imaging and has extraordinary sensitivity towards harmonic generation. SHG data suggests the presence of more fibrillar collagen in the diseased kidneys. The combinations of short wavelength FLIM and SHG analysis results in a robust analysis procedure independent of observer interpretation and let us create a criterion to quantify the extent of fibrosis directly from the image. The progression of fibrosis in UUO model has been studied using this new FLIM-SHG technique and it shows remarkable improvement in quantification of fibrosis compared to standard histological techniques.

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Introduction

Pathologically, chronic kidney disease (CKD) 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 CKD, many pre-clinical 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 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 inter- and intra-observer variability when visual assessment is used to quantify collagen.[3,4] Recent data has 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.[6–7] 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 DIVER (Deep tissue Imaging via Enhanced photon Recovery) system uses forward detection and exhibits extraordinary sensitivity to SHG. [8,10–12]

Fluorescence imaging of fibrotic tissues typically involves extrinsic fluorescent labeling and autofluorescence imaging with a two 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 non-zero 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 analysis to obtain fibrosis scores from an image in an automated fashion without any operator intervention.

We hypothesized that label free quantification of fibrosis using SHG FLIM would correlate well with Sirius Red imaging of diseased mouse kidneys after UUO.

Results

To validate SHG and FLIM in a mouse model of renal fibrosis, we chose the widely used unilateral ureteral obstruction (UUO) model. We used uninjured left kidneys as controls, a

strategy that is commonly employed. Picrosirius Red-stained sections of injured and uninjured kidneys were viewed using conventional bright field microscopy and polarized microscopy and showed a time-dependent increase in collagen deposition (Figures 1a–c). Masson Trichrome images of 7-day post-UUO kidneys revealed a doubling of collagen deposition. (Figures 1d–e).

The evolution of the SHG signal with time is shown in Figures 2a, 2b and 2c. The plot of the SHG signal as a function of time of UUO is shown in Figure 2e and Supplementary Figure S4. SHG imaging showed an 11-fold increase in collagen content at 21 days post-UUO while Picrosirius Red Staining showed a four-fold increase (Figures 1c and 2e). We next performed 3D SHG imaging in 100 μm thick sections to acquire a more comprehensive view of tissue fibrosis than is seen with 5 μm sections used for histology (Figure 3). These images were reconstructed after background correction and show that there are significantly more collagen fibers localized around the glomerulus for the 21 day (most fibrotic) right kidney (Figure 3a) to the 7 day (least fibrotic) left kidney (Figure 3b).

We next quantified fibrosis using a combined SHG and FLIM approach. The rationale behind this approach is that disease progression is associated with accumulation of collagen in the tissue as well as loss of tissue architecture. Fibrillar collagen is primarily responsible for creating the SHG signal and is represented in green in the phasor-masked images. The FLIM images displayed in red are measurements of cells within the kidney. Hence the increase of ratio of f_{green}/f_{red} correlates with increasing fibrosis and decreasing renal tissue.

The phasor masked intensity images for the 7, 14 and 21 days of UUO are colored according to the scheme presented in the phasor plot in Figure 4d and the representative images are shown in Figures 4a, 4b and 4c, respectively. The increase of the f_{green}/f_{red} ratio with the progression of the disease is shown in Figure 4e. The trend line created by fitting the data with a polynomial shows that this method can help distinguish this difference at a very early stage of the disease, potentially even before 7 days. The ratio of f_{green}/f_{red} also increases mildly for the left kidney. This may represent hyperfiltration of the solitary kidney and could reflect a limitation of using contralateral kidneys as controls.

The f_{green}/f_{red} ratio analysis does not involve any operator intervention, as there is no background correction from the intensity histogram. Thus these images can be analyzed in a completely automated way. In Picrosirius Red imaging, compared to the 7-day control kidney the 21-day diseased kidney shows 9 fold increase in signal. In the f_{green}/f_{red} method the same signal increase is 20.4 times (Supplementary Figure S1 and S2).

Discussion

In this study, we have found that imaging of fibrotic kidneys using a combined FLIM-SHG approach is a very sensitive indicator of disease progression and fibrosis in the mouse UUO model. Analysis of forward-propagated SHG alone sensitively detects collagen deposition and can be combined with 3D reconstruction in thick tissues to sample a greater area. Furthermore, a novel technique employing both SHG and FLIM was shown to be a sensitive indicator of renal disease progression. Previous studies in other mouse models have

evaluated SHG as a technique to measure renal fibrosis but did not compare sensitivity between the techniques.[6,7] Our studies focused on the UUO model of renal fibrosis since it is one the most widely-used models of tubulointerstitial fibrosis.

This is the first study to combine FLIM with forward-propagating SHG to measure renal disease progression. Previous fluorescent imaging of kidneys commonly used two-photon excitation which relies on fluorescent intensity. Since FLIM measures the decay rate of the fluorescent signal, it may represent a better and more specific technique to measure tissue autofluorescence.

In summary, we have shown that combined SHG-FLIM imaging is a sensitive and accurate technique to measure the heterogeneous process of tubulointerstitial fibrosis. This can be done in an automated fashion and can quickly provide a thorough evaluation of renal disease progression. Although multiphoton lasers are expensive and not universally available, the decreasing cost of single wavelength femtosecond lasers indicates that this technique could be potenteialy be widely adopted. This technique would likely be used in conjunction with standard histologic techniques and could be modified to measure collagen in an entire kidney as was recently reported.[18] Future studies will be needed to determine the applicability of these techniques for live animal imaging as well as quantification in other models of renal fibrosis.

Methods

Details of UUO surgery, slide preparation, histologic studies, FLIM analysis and DIVER setup are available in the supplemental methods.

Analysis of the Second Harmonic Generation and short wavelength fluorescence

The type of measurement that shows the maximum difference between the relatively healthy left kidney and the diseased right kidney over the progression of the UUO was the SHG channel data acquisition, when analyzed without background subtraction. The optical filters used in this channel, combination of BG39 (blue (2) in Figure S1) and UG11 (black (1) in Figure S1), is meant to primarily separate SHG signal (green (4) in Figure S1). In these measurements the samples were excited with 710 nm excitation laser line using a two-photon excitation scheme. The optical filter can pass short wavelength fluorescence from 360 nm to 410 nm (light blue shaded area in between UG11 (black) and BG39 (blue) in Figure S1). To identify the origin of this short wavelength fluorescence signal the average fluorescence spectra of the tissue samples were measured using an Olympus FV1000 microscope equipped with a grating-based spectrograph (Andor SR303i) with a 512-channel ultrafast EMCCD (Andor iXon Ultra). The optical restrictions associated with the Olympus FV1000 microscope set up prohibit measurements below 360 nm.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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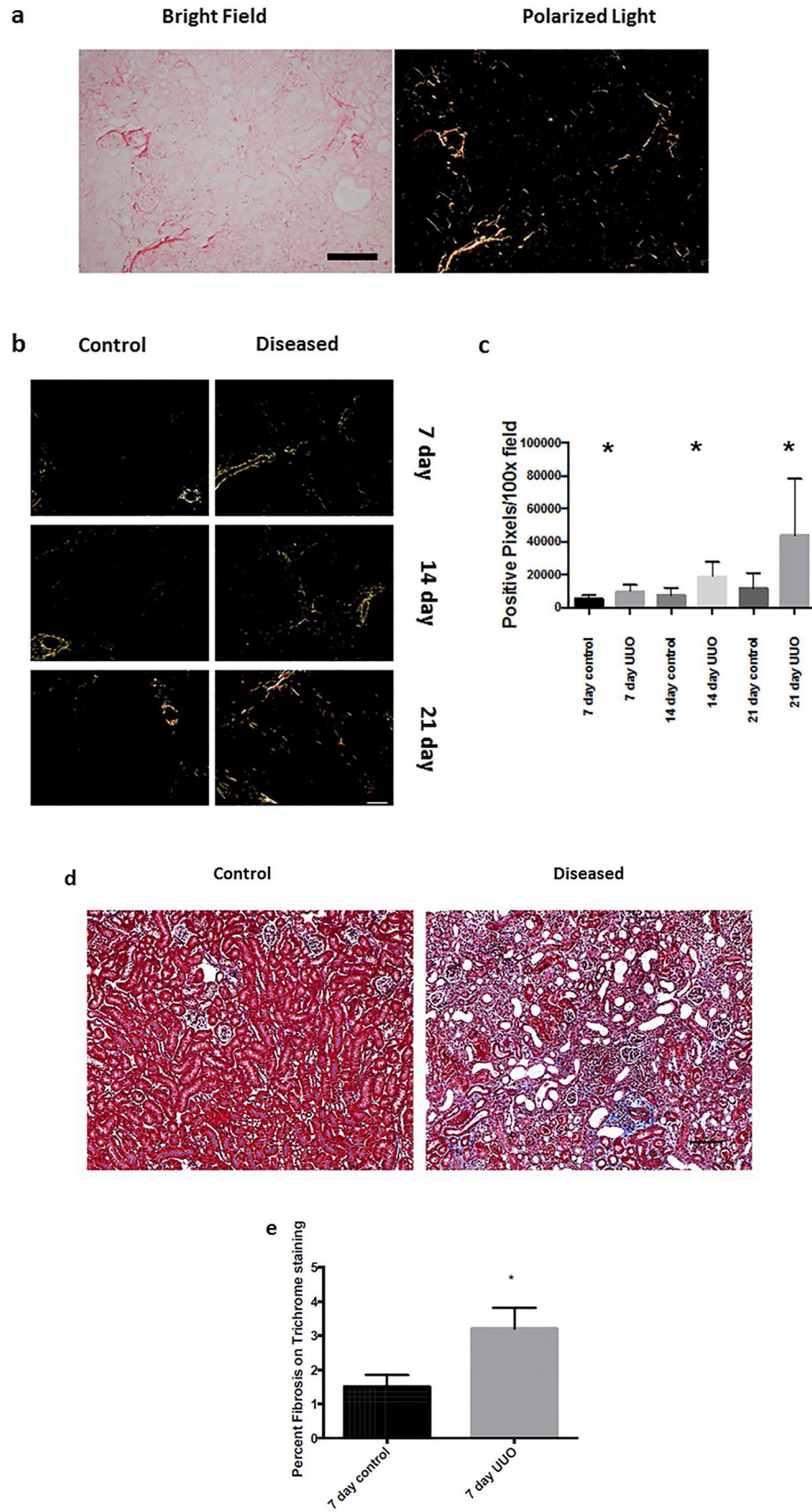
a

b

c

d

e

Figure 1. Quantification of Fibrosis Using Histologic Methods

Picrosirius Red-stained kidneys were visualized under both bright field microscopy and polarized light microscopy (a–c). Figure 1a demonstrates representative 100x images of the same section visualized with both techniques. There is a time-dependent increase in collagen deposition seen using polarized light microscopy (Figures 1b–1c).* $p < 0.05$ vs. control at same time point. Masson Trichrome staining was also performed and revealed tubular damage and collagen deposition (d). 100x representative images are shown. Seven days post-UUO, there are a significant number of dilated tubules (arrowhead) and collagen fibrils (arrow). Quantification of collagen shows a significant increase seven days post-UUO. * $p < 0.05$ vs. control. Scale bar = 25 μm.
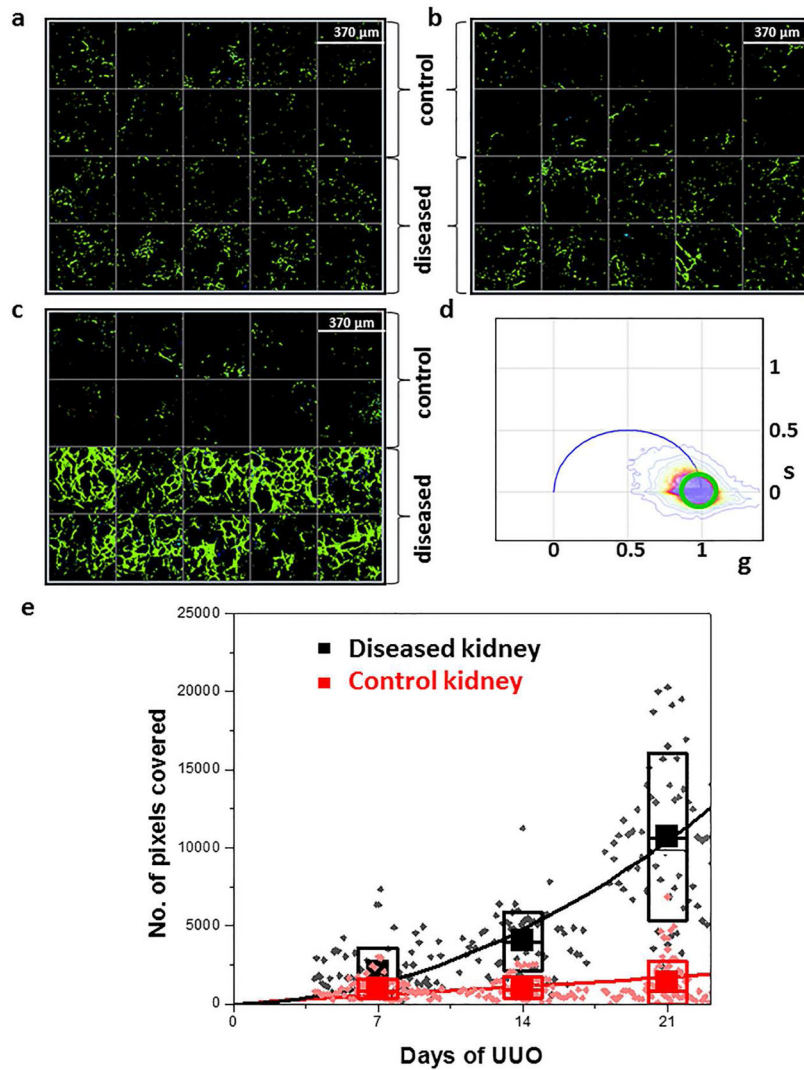
Figure 2. The evolution of the area covered by SHG signal as a function of days of UUO

Figures 2a, 2b, 2c show the phasor images of SHG signals generated from mouse kidneys of 7, 14 and 28 days of UUO, respectively. The top two rows (first ten images) in each of these panels show the data acquired for left kidney and the bottom two panels shows the SHG images for the right kidney. The phasor points originating from these measurements are shown in Figure 2d, where the green cursor was used to select the SHG signal appearing at s=0, g=1. Figure 2e shows the progression of the SHG as a function of days of UUO. The black and red in this figure represents the diseased right kidney and normal left kidney, respectively. The filled squares show the mean of the measurement and the boxes show the ± SD of the measurements. The values of individual measurements from the left and right kidney are plotted in dark grey and light red. The trend lines, created using a polynomial fit, show the progression of disease in both the kidneys. The p value of the Student's T test at each time point of the disease is <0.0001.
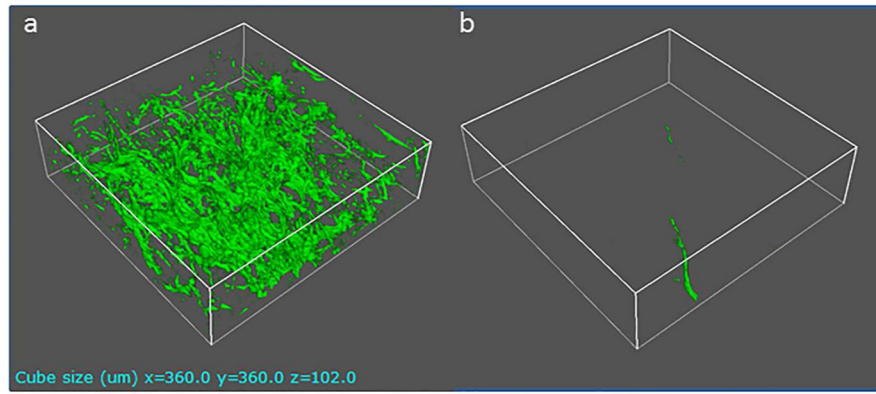
Figure 3. 3D image of collagen fibers originating during course of UUO

3D image from 21 day right kidney, the most fibrotic one **(a)** and 7 day left kidney, the least fibrotic one **(b)** are shown. The two images exemplify the different amount of collagen during the progression of the disease. These images were acquired from 100 Nm slices with a 360 Nm FOV for every 2 Nm of depth.
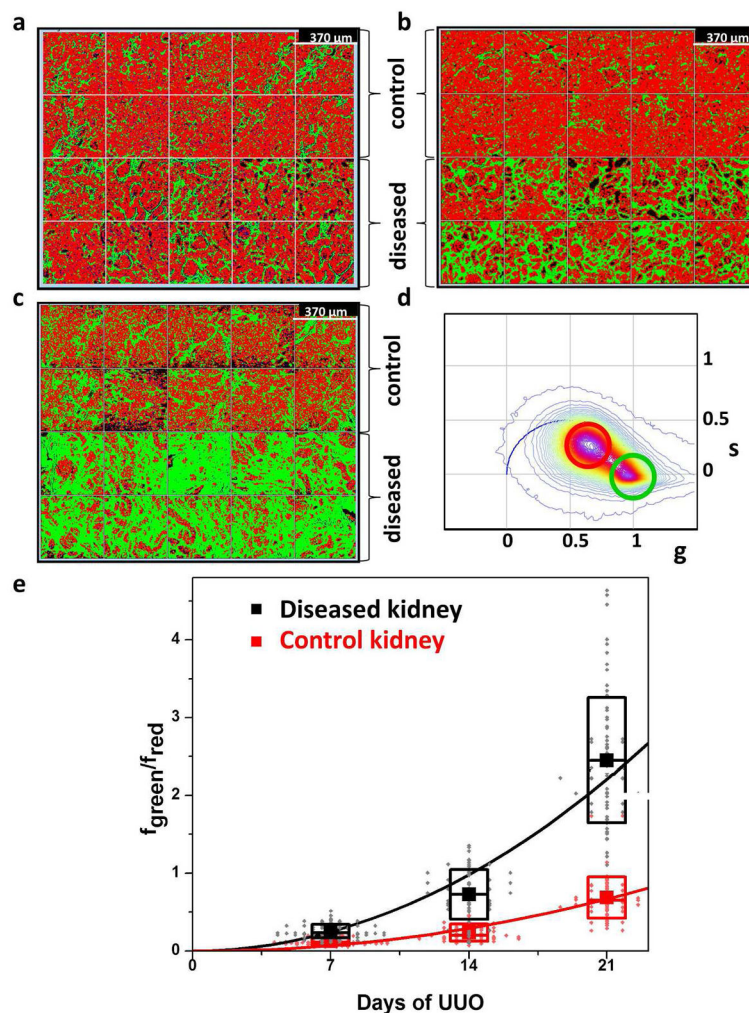
Figure 4. The evolution of the ratio of f_{green}/f_{red} as a function of days of UUO

Figures 4a, 4b, 4c represent the phasor masked images, colored using the cursor colors in the phasor plot of Figure 4d, from mouse kidneys of 7, 14 and 28 days of UUO, respectively. The top two rows (first ten images) in each of these panels show the phasor masked images of the left kidney and the bottom two panels shows the images for the right kidney. THE FOV for each image was 370 Nm. In the phasor plot (Figure 4d), the green cursor were used to select the SHG signal appearing at s=0, g=1; and the red cursor was used to select the short wavelength fluorescence that can bleed through the filter and have a non-zero lifetime. Thus the pixels in the image whose origin is SHG are covered by green and pixels where the origin is bleed-through autofluorescence are covered by red color. Figure 4e shows the progression of the ratio of fraction covered by green to areas covered by red (f_{green}/f_{red}) as a function of days of UUO. The black and red in this figure represents the diseased right kidney and normal left kidney, respectively. The filled square shows the mean of the measurement and the box shows the ± SD of the measurements. The values of individual measurements from the left and right kidney are plotted in dark grey and light red. The trend

lines, created using a polynomial fit, show the progression of disease in both the kidneys. The p value of the Student's T test at each time point of the disease is <0.0001.