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Phasor approach to autofluorescence lifetime imaging FLIM can be a quantitative biomarker of chronic renal parenchymal injury.

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Abstract

Diabetic kidney disease continues to be the leading cause of chronic kidney disease, often advancing to end stage kidney disease. In addition to the well characterized glomerular alterations including mesangial expansion, podocyte injury, and glomerulosclerosis, tubulointerstitial fibrosis is also an important component of diabetic kidney injury. Similarly, tubulointerstitial fibrosis is a critical component of any chronic kidney injury. Therefore, sensitive and quantitative identification of tubulointerstitial fibrosis is critical for the assessment of long-term prognosis of kidney disease. Here, we employed phasor approach to fluorescence lifetime imaging, commonly known as FLIM, to understand tissue heterogeneity and calculate changes in the tissue autofluorescence lifetime signatures due to diabetic kidney disease. FLIM imaging was performed on cryostat sections of snap-frozen biopsy material of patients with diabetic nephropathy. There was an overall increase in phase lifetime (τ_{phase}) with increased disease severity. Multicomponent phasor analysis shows the distinctive differences between the different disease states. Thus, phasor autofluorescence lifetime imaging, which does not involve any staining, can be used to understand and evaluate the severity of kidney disease.

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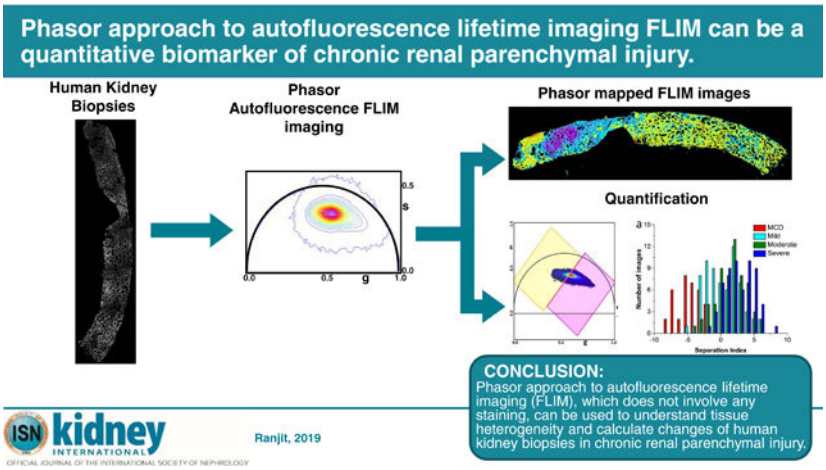
Disclosure

The authors have no competing financial interests.

Graphical abstract



Keywords

Tubulointerstitial fibrosis; NADH; Autofluorescence; FLIM; Phasor

Introduction

Diabetic nephropathy continues to be the leading cause of chronic kidney disease, often advancing to end stage renal disease. In addition to the well characterized glomerular alterations including mesangial expansion, podocyte injury, and glomerulosclerosis, tubulointerstitial fibrosis is also an important component of diabetic kidney injury[1–4]. Similarly, tubulointerstitial fibrosis is a critical component of any chronic renal injury[5]. Therefore, sensitive and quantitative identification of tubulointerstitial fibrosis is critical for the assessment of long-term prognosis of diabetic and nondiabetic kidney disease, and has implications for therapeutic decision-making.

Our goal in this project is to use autofluorescence generated from the intrinsic fluorophores that are present in diabetic kidney tissue biopsies to calculate the degrees of the disease. Autofluorescence from cells and tissues have multiple sources including NADH and FAD[6]. The changes in NADH and FAD fluorescence properties are indicative of changes in metabolism and have been used to study cancer, nonalcoholic steatohepatitis, Alzheimer's and other diseases[7–12]. Most of these studies involve measuring changes in fluorescence intensities, which is dependent on concentration and the changes in quantum yields. The quantum yield is dependent on the environment and the physical state of the fluorophore. NADH quantum yield changes 9 times from free to protein bound state as the collapsed NADH structure expands in bound state and the adenine and nicotinamide rings separate[13]. Compared to free NADH, equal concentration of bound NADH can give 8 times more fluorescence. FAD quantum yield is also dependent on its biding state. Changes in the fluorescence intensities can thus be either due to changes in concentration or their physical states, and this makes separating these two effects difficult. To circumvent this problem fluorescence lifetime has often been employed as the choice of measurement. Lifetime is

independent of the concentration and is only dependent on the different species and their states, and can give insight into the molecular species present[12]. In tissue samples, collagen along with NADH and FAD can contribute towards fluorescence, as collagen fibers have overlapping spectra with NADH and FAD.

In this work we have used the phasor approach to fluorescence lifetime imaging (FLIM) to characterize and analyze the autofluorescence signals obtained from frozen kidney biopsy tissues[14, 15]. The excitation wavelength (710 nm) and the choice of the emission window ensured that the main fluorescent species responsible for the fluorescence are the free and bound NADH[15, 16]. The details of the phasor approach have been explained elsewhere and in the supplemental information[15]. Briefly, phasor approach is a fit free method where the fluorescence decay information from each pixel is transformed to a point in the phasor plot and its position is determined by the decay information. Pixels with shorter average lifetimes have smaller phase angles and longer lifetimes have larger phase angles. This is exemplified in Figure 1. Phasor analysis is fit-free and is much faster compared to the traditional iterative fitting approaches. Another aspect of the phasor approach is the reversibility principle of the image analysis. A set of points in the phasor image can be selected and the intensity image can be colored accordingly. Alternatively, an area of an image can be selected and their positions in the phasor plot can be highlighted to study the similarity of the decay parameters. The phasor approach has been used to understand cancer, steatosis, fibrosis in tissues and recently has been expanded to identify cell types in mice kidneys[8, 15–19].

To our knowledge this approach and autofluorescence of tissues have never been expanded to understand human kidney disease and specially to characterize disease states. The following analysis can be automated, eliminating operator bias.

Results

The phasor plot from kidney tissue biopsies has a large lifetime distribution. The first important observation is the increasing phase lifetime with increasing disease severity. This is observed by the changes in the phasor cloud positions. Using the reversibility principle of the phasor plot, the samples were colored using multiple cursors. In minimal change disease (MCD) samples, the images are mostly purple and cyan. In the mild disease, the color in phasor mapped image changes to more yellow. In moderate disease it is mostly yellow and red started appearing and in severe diseases it is mostly red. These images are pseudo-colored and the color in FLIM images represents the corresponding cursor color in the phasor plot used for selection. The data clearly suggests that there is a change in the phasor-FLIM signatures with changes in disease state.

The images in Figure 2 shows the heterogeneity in autofluorescence lifetimes and applicability of phasor-FLIM to understand the changes in tissue composition. The representative phasor plots can be seen in supplemental figure S5. However, to create a method of calculating the extent of the disease, we used a ratiometric approach, similar to our work in calculation of fibrosis in the UUO model[16]. Briefly we split the phasor distribution in two areas, yellow representing longer phase lifetimes and purple representing shorter phase lifetime (Figure 3) and the images were colored accordingly. The ratio of the

area covered by yellow to purple was used for the analysis (Supplementary Figure S6). An important aspect of this analysis is that the scores from individual areas can be seen and compared in each field of view (Figure 3). Pathologists can then look at the areas important for the disease. Averaging the whole image decreases the sensitivity of the measurement and this quantification is shown in Supplementary Figure S6. The data shows MCD (black bar) can be easily separated from the mild (green), moderate (blue) and severe (red) diabetic kidney disease states. There is a gradual increase according to the severity of the disease.

The ratiometric analysis simplifies the changes that is seen in the images. However, this simplification ignores the high amount of complexity in the images. The images are not homogeneous and this inhomogeneity (see supplemental figures) can be used to see different areas of the biopsies and compare the lifetime signatures from those specific areas.

The multiparametric phasor analysis was used to see the difference in lifetime signature of the different states are different or not.[12] This analysis takes a much deeper look at the phasor signature of the images. This analysis can show the gradual changes in the phasor signatures. The overall changes are shown in Figure 4. Figure 4a shows that the MCD (red histogram) can be very well separated from the severe injury (blue injury). This is also observed in the receiver operating characteristic curve (ROC, Figure 4b). The images from the mild (cyan) and moderate (green) show that there is a continuous change in the phasor properties. Histograms of individual images are shown in supplemental figures S9 and S10. The very large variation in autofluorescence signature can be observed from these images. Overall, the analysis shows that for mild and moderate disease states, the phasor autofluorescence signature is different from that of the severe disease states in certain areas of the samples and resembles that of the severe in other areas.

Discussion

The analysis shows the applicability of the FLIM imaging in distinguishing tubulointerstitial fibrosis. The ratiometric analysis simplifies the method but disregards one critical aspect of the imaging – the inhomogeneity of the fluorescence lifetimes in different parts of the image and in between different samples. One of the main rationales for the use of imaging techniques is the ability to understand spatial information and separation of those spatial information. In contrast traditional analysis only uses an average calculation. Thus, overall calculations, while simplifying the understanding of the system, ignores the detailed map. This information and the heterogeneity is inherent in the FLIM images and can be observed by choosing a small section of the image and restricting the calculation in that section. An example of these types of calculations, even in a simple ratiometric way is shown in Figure 3, where the disease scores are shown in each individual area. A more in-depth analysis shows the diversity in autofluorescence signature increasing in different parts of the image for the mild and moderate disease states. Multiparametric phasor analysis can separate the MCD and severe disease states and mild and moderate states lie in the middle. The ratiometric analysis of phasor plots is visual and gives a direct perceptible understanding to the images. This can be combined with traditional histology to understand samples deeper. Multiparametric analysis is much deeper and being a machine learning type approach, the differences are deeper but not as visual.

Combined with traditional pathology, FLIM can provide insight to the molecular changes along with changes in the structures. These molecular insights are lacking in traditional histology. Combination of FLIM and phasor approach results in a fit free fast analysis scheme and the DIVER microscope enables imaging of these large area images ~ (1.5 cm × 1.5 cm). In our knowledge this is the first time this autofluorescence imaging method, without the need of labeling, has been employed to study tubulointerstitial fibrosis in human kidney biopsies and has a great road ahead with a potential towards diagnostic capabilities.

Methods

Tissue procurement and sectioning

Following institutional review board approval, we searched the University of Chicago pathology archives from 2015–2016 for kidney biopsies with a diagnosis of diabetic nephropathy (DN). The sections had been snap-frozen and cut at 4–5 μm thickness in a cryostat. DN was defined as mild, moderate, or severe based on the type of glomerular lesions and extent of interstitial fibrosis and tubular atrophy (IFTA).[3] The details about the classification is explained in supplemental material methods.

Imaging and FLIM phasor analysis

The details of the phasor approach to FLIM and imaging using DIVER microscope are explained in the supplemental material.[14, 15, 20] In short DIVER enables us to automatically image a large area and FLIM phasor analysis results in a fit free fast analysis scheme. In Figure 2, the phasor cloud has been selected by multiple cursors of equal angles. The images were colored accordingly. The data acquisition and analysis were controlled by SimFCS software developed at Laboratory for Fluorescence Dynamics, University of California Irvine.

Ratiometric analysis

The ratiometric analysis enables us to quantify the changes and see the overall change in the tissue signatures. This was done by splitting the phasor cloud into two and calculating their relative ratios. This is similar to the ratiometric analysis we employed before to characterize fibrosis in mice kidneys. The longer and shorter lifetimes were selected using a yellow and a purple cursor, respectively. We used inclined cursor with 15-unit width, 10-unit height and 50° inclination. The disease ratio for each imaging area was calculated using the following formula:

$$Diseaseratio = \frac{f_{yellow}}{f_{yellow} + f_{purple}} \quad (1)$$

Where, f_{yellow} and f_{purple} are the fraction of the pixels covered by yellow and purple, respectively. The average disease ratio was calculated using the following:

$$Diseaseratio_{av} = score = \frac{\sum N_{pixels} \times Diseaseratio}{\sum N_{pixels}} \quad (2)$$

$\sum N_{pixels}$ is the total number of pixels in each field of view (FOV).

These average disease ratios are plotted in Supplemental Figure S6. The ratios from each individual FOVs are shows in Figure 3.

Multiparametric analysis of phasor distribution

We have also used multiparametric phasor distribution to understand if the phasor and lifetime signatures are indeed different between MCD and different disease states. Multiparametric analysis has been explained elsewhere and is explained in detail in the supplemental material.[12] Briefly, this analysis involves splitting the phasor histogram vertically in four sets based on the number of points can then calculates a series of shape and positon parameters. These shape parameters are combined to create a spectrum specific to the particular image. Finally, a fitting using an arbitrary wait for these shape parameters separates the control and the most diseased groups. Then the extent of disease states are calculated based on these weighing parameters used on their spectra.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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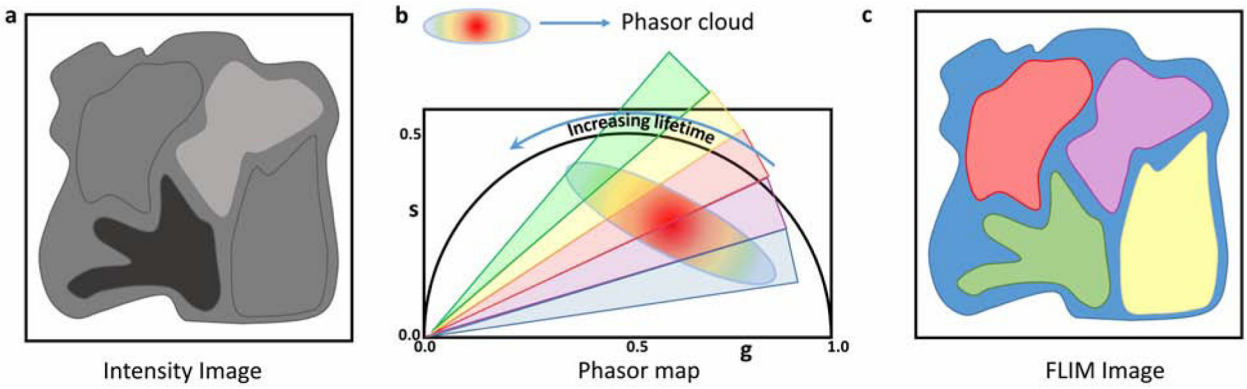

Figure 1: Phasor approach to FLIM.

(a) Intensity image and (b) the corresponding phasor plot. The Phasor cloud can be selected using cursors and the corresponding areas that the phasor cloud originated from can be colored accordingly in a FLIM image (c). These areas can be either same in intensity (similar grayscale in a) or of different intensities (different grayscales). The phase angle in phasor plot (b) increases with increasing lifetime and increasing lifetimes can be selected by using angular cursors.

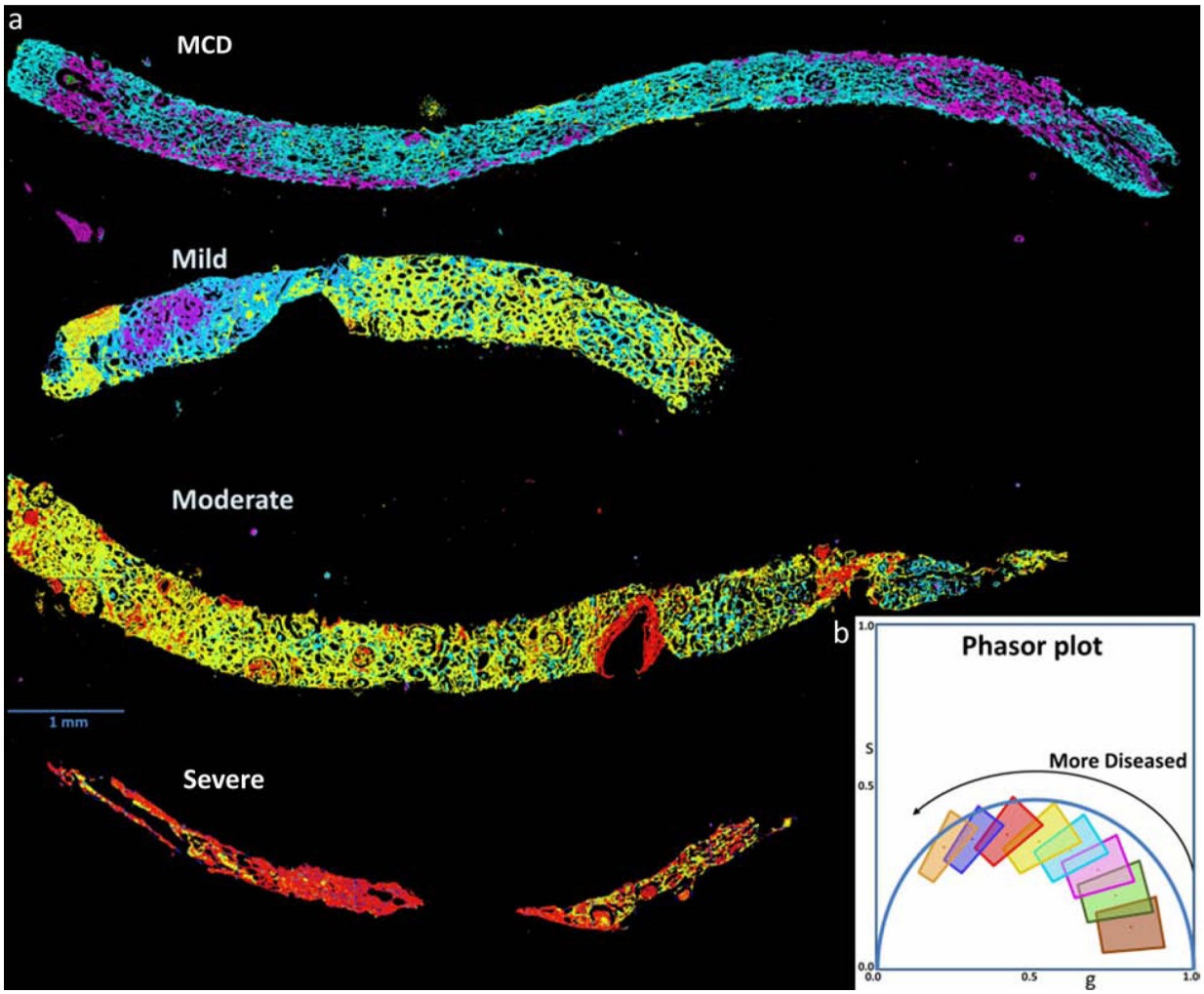

Figure 2. Phasor mapped FLIM images originating from human kidney biopsies

(a). The images were colored according to the cursors whose positions are shown in the phasor plot (b). The data shows increase in phasor lifetime with increasing severity of the disease.

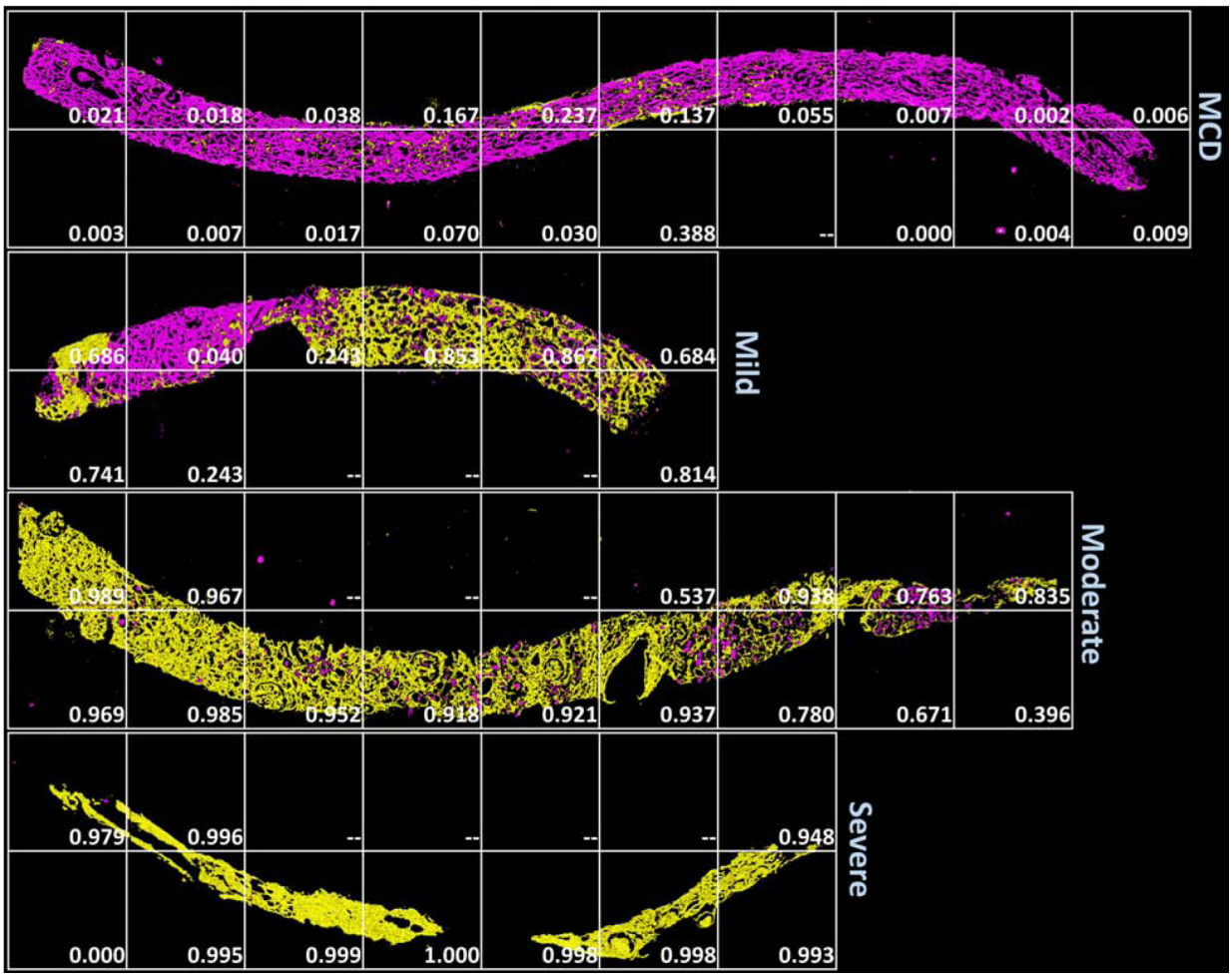

Figure 3. The changes in the tissue architecture as observed by the two-fraction analysis.

Here the phasor plot was split into two (yellow and purple) and the images were colored accordingly. The images show that different parts of the image can have variability in behavior. The values at box is the score at that area. Each box size − 970 μm.

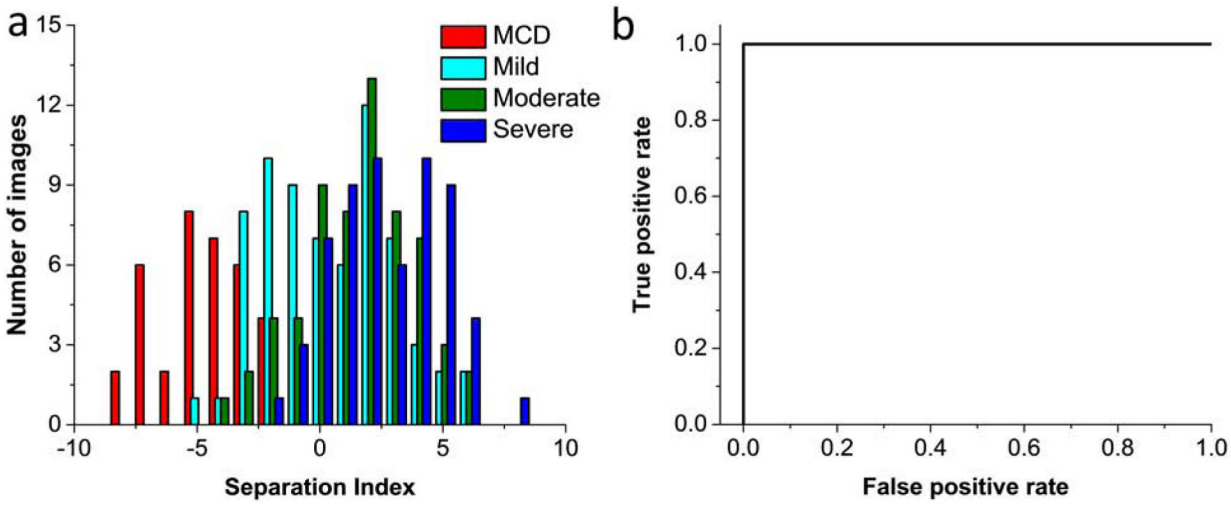

Figure 4. Multiparametric phasor analysis of kidney disease.

(a) Multiparametric phasor analysis shows that the spectra obtained from the phasor plots of the MCD (red histogram) samples can be easily separated from the spectra of the phasor plot of severe disease states (blue histogram). The mild (cyan) and moderate (green) histograms shows that there is a continuous change in phasor signature. (b) The receiver operating characteristic curve (ROC) shows the high separability that can be achieved using this method.