A portable fiberoptic ratiometric fluorescence analyzer provides rapid point-of-care determination of glomerular filtration rate in large animals

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Measurement of the glomerular filtration rate (GFR) is the gold standard for precise assessment of kidney function. A rapid, point-of-care determination of the GFR may provide advantages in the clinical setting over currently available assays. Here we demonstrate a proof of principle for such an approach in a pig and dogs, two species that approximate the vascular access and GFR results expected in humans. In both animal models, a sub-millimeter optical fiber that delivered excitation light and collected fluorescent emissions was inserted into a peripheral vein (dog) or central venous access (pig) by means of commercial intravenous catheters. A mixture of fluorescent chimeras of a small freely filterable reporter and large non-filterable plasma volume marker were infused as a bolus, excited by light-emitting diodes, and the in vivo signals detected and quantified by photomultiplier tubes in both species in less than 60 min. Concurrent standardized 6-h iohexol plasma kidney clearances validated the accuracy of our results for both physiologic and a chronic kidney disease setting. Thus, our ratiometric technique allows for both measurement of plasma vascular volume and highly accurate real-time GFR determinations, enabling clinical decision making in real time.

Kidney International (2012) 81, 112-117; doi:10.1038/ki.2011.294; published online 31 August 2011

KEYWORDS: acute kidney injury; chronic kidney disease; glomerular filtration rate

Quantifying kidney function and/or injury remains a vexing and increasingly important clinical problem. This results in the inability to diagnose and stratify patients with acute kidney injury or chronic kidney disease appropriately, follow progression of the disease, or response to therapy.¹-⁴ Measurement of glomerular filtration rate (GFR) is the gold standard for precise assessment of kidney function.¹ There are many well-known potential clinical applications in which GFR measurement could directly improve patient management. For example, GFR may be beneficial while determining drug-dosage adjustments in patients with acute or chronic kidney dysfunction, as a screening test for hospital-acquired kidney injury in patients with or without preexisting kidney damage, to assess therapeutic response or rate of progression of kidney disease, to monitor patients requiring repeated administration of nephrotoxic drugs, and potentially as an indirect assessment of visceral organ perfusion, such as during prolonged anesthesia.¹-³,⁵ Despite these potential roles for GFR determination in optimization of patient care, quantification with validated GFR markers is uncommonly performed in the clinical setting because existing GFR measurement techniques require several blood draws over an extended period of time, administration of markers that require specialized preparation, handling (that is, radionuclides), or assays (that is, inulin and iohexol), or submission of samples to external laboratories.¹

Attempts have been made using serum creatinine or cystatin C to estimate GFR, the so-called estimated GFR; however, accuracy, the dependence on a stable serum creatinine, and decreased reliability outside of a narrow range of 20-60 (ml/min per 1.73 m²) have greatly limited the utility of this approach. In addition, the necessity of having quantitative analysis of GFR in therapeutic trials is well established.⁶ Therefore, we hypothesized that the development of a point-of-care bedside fluorescent technique, with in vivo rapid detection and instantaneous calculation of GFR,
would allow for the rapid, reproducible, inexpensive, safe, and patient-friendly determination of GFR in a time frame necessary to make appropriate clinical decisions. We demonstrate here that the portable analyzer developed for this purpose allowed point-of-care determination of GFR within 60 min of administration of the fluorescent conjugates, with minimal adverse effects and excellent agreement with the 6-h iohexol-based GFR technique. Our results provide proof of principle for the use of this ratiometric analyzer for rapid determination of GFR in larger species that approximate the vascular access and GFR results expected in humans. The principle governing this unique ratiometric fluorescent approach to quantifying kidney function can be expanded to other organs and many other processes allowing for improved accuracy and far more rapid clinical decision making.

RESULTS
Design of the ratiometric optical fiber system
Translation of our previously described two-photon technique to the clinical situation required development of a system capable of generating and delivering two excitation sources and quantifying the detection of the corresponding emissions. The ratiometric fiber optical system developed contained blue (center peak 490 nm, full width half maximum 30 nm, Roithner Laser Technik) and amber (center peak 590 nm, full width half maximum 20 nm) light-emitting diodes (LEDs), which provided excitation illumination for fluorescein (green) and Texas Red fluorescent conjugates, respectively (Figure 1a and b). A condenser, consisting of a pair of plano-convex lenses, placed after each LED provided a nearly collimated beam, and stray light was reduced with apertures placed both before and after the condensers. The excitation bandpass filters were Semrock FF475/20 and FF579/34 for the blue and amber LEDs, respectively. After passing through these designated excitation filters, the two nearly collimated beams were merged at the excitation dichroic mirror (Semrock FF520-Di01) that reflects blue and transmits amber. A dual-band dichroic mirror (Semrock FF505/606-Di01) then reflected the merged excitation light 90° toward a biconvex fiber lens (LB1761-A, Thorlabs) that focused the beam onto a 0.75 mm optical fiber placed behind

Figure 1 | Design of the ratiometric optical fiber system. (a) Optical layout. 1a and 1b, light-emitting diodes (LED) power supplies; 2a and 2b, amber and blue LEDs, respectively; 3a and 3b, collimating condensers with apertures; 4a and 4b, excitation bandpass filters; 5, excitation dichroic mirror; 6, dual-band dichroic mirror; 7, fiber lens with aperture; 8, fiber adapter; 9, optical fiber; 10, catheter; 11, emission dichroic mirror; 12a and 12b, photomultiplier tube (PMT) focus lenses with apertures; 13a and 13b, emission filters; 14a and 14b, PMTs. (b) Cross-section showing optical components. (c) Electronic system of the device: SPI, serial peripheral interface.
Detection of GFR in large animals

Fluorescence-vs.-time curves were successfully generated in a pig for both the small and large dextran conjugates (Figure 2). These fluorescent conjugates have previously been used in an in vivo rat model using two-photon microscopy. Although the signal from the large nonfilterable plasma volume ‘marker’ dextran (red channel) remained stable over time, the signal of the filterable 5 kDa fluorescein isothiocyanate (FITC)-inulin (green channel) ‘reporter’ initially decreased rapidly because of combined inter-compartment redistribution (movement into the extracellular space) and kidney clearance, followed by the later slower decrease in fluorescence solely due to kidney clearance. The normalized fluorescence ratio-vs.-time curve produced by calculation of the green/red ratio is shown in Figure 2 along with the raw data from both channels shown in the figure inset. Plasma volume, determined by dilution of the large dextran molecule, was 1012.3 ml, or 3.4% of total body weight. Calculated GFR from the serial iohexol samples was 2.02 vs. 2.11 ml/min/kg as determined using the ratiometric two-compartment method in 60 min.

Fluorescence-vs.-time curves were also reproducibly generated in dogs for both the small and large dextran conjugates with peripheral venous access via the cephalic vein. An example of a dog under physiological conditions with mild sedation (see Materials and Methods) is shown in Figure 3a. More frequent and higher spike artifacts, which were reduced in the green/red ratio curve, corresponded to movement by the non-anesthetized dog during data collection. An example of a dog with a reduced GFR, anesthetized using isoflurane, is presented in Figure 3b, showing a slower fluorescence ratio decay, an indication of a slower kidney clearance, compared with the curve from the normal dog shown in Figure 3a. More frequent and higher spike artifacts, which were reduced in the green/red ratio curve, corresponded to movement by the non-anesthetized dog during data collection. An example of a dog with a reduced GFR, anesthetized using isoflurane, is presented in Figure 3b, showing a slower fluorescence ratio decay, an indication of a slower kidney clearance, compared with the curve from the normal dog shown in Figure 3a. Table 1 shows GFR values obtained using three different techniques from four different dogs, three of which had normal kidney clearance. The values contained in the bottom row were generated from a dog with chronic kidney disease. Plasma volumes obtained using the large dextran were also shown in the table. No systemic toxicity was noted in these studies.

**DISCUSSION**

Translation of fluorescent technology into quantitative in vivo clinical applications has been rare, despite the extremely high sensitivity of fluorescence techniques, because of its complex and platform-dependent nature. As a result, accurate determination of the concentration of fluorescent molecules under in vivo conditions is usually very difficult. With this in mind, we felt that the ideal starting point for clinical translation of fluorescent technology was with the quantification of GFR using the ratiometric technique. In addition to being able to use inert and highly water-soluble molecules, such as dextrans, for our parent molecules, the fluorescent labeling of dextrans is well worked out and offers high conjugation ratios. This allowed for very small amounts of fluorescent dextrans to be used in our studies, and has
The rapid and accurate determination of GFR, the primary functional marker for the kidney, has been a clinical goal for over 50 years. The cumbersome, time-consuming, and expensive techniques presently available have not met this important clinical need. Development and use of estimating formulas based on serum creatinine or cystatin C have been disappointing and also have not met this need for several well-explained reasons. Therefore, the development of a rapid, accurate, safe, easy, and inexpensive technique has high clinical importance both in and outside of the hospital.

Following up on our previous two-photon microscopic rat studies, we have now translated our approach to large animals with the development of an optical catheter and fluorescent analysis device. We have demonstrated that using this novel method employing inert fluorescent size-specific dextran molecules we were able to accurately, reproducibly, and rapidly quantify the GFR in dog and pig models in real time at the point of contact. The GFR values obtained using the device showed excellent agreement with that from the established iohexol approach that required 6 h of repeated plasma sampling and extensive laboratory processing before GFR calculations. We also demonstrated that the technique was accurate in a dog with severely reduced kidney function.

**Table 1 | Normal and CKD GFR of dogs**

<table>
<thead>
<tr>
<th>Dog ID</th>
<th>GFR (ml/min/kg) by spectroscopy</th>
<th>GFR (ml/min/kg) by device</th>
<th>GFR (ml/min/kg) by iohexol</th>
<th>Condition</th>
<th>Weight (kg)</th>
<th>Plasma volume (ml)</th>
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<td>4.4</td>
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<td>1.19</td>
<td>1.45</td>
<td>CKD</td>
<td>29.8</td>
<td>1482</td>
</tr>
</tbody>
</table>

Abbreviations: CKD, chronic kidney disease; GFR, glomerular filtration rate.

GFR values obtained by three different techniques along with plasma volumes from three normal dogs and one CKD dog are shown. During the tests dogs 1 and 2 were with mild sedation with 0.1 mg/kg intravenously administered acepromazine; dogs 3 and 4 were anesthetized using isofluorane.

**Figure 3 | Quantifying plasma volume and glomerular filtration rate in dogs using fluorescent dextrans.** The data shown in a and b were generated from a dog under physiological conditions and a chronic kidney disease model, respectively. The 5/150 kDa ratio data (dots) from the optical device were fit to a bi-exponential equation. In each case, the fitted curve is shown as a solid line. A good overlap is seen between the device data points and the plasma fluorescence of the 5 kDa fluorescein isothiocyanate-dextran (triangles) from a spectroscopic analysis. The original data that generated the ratio are shown in the inset in each panel.
There are several major advantages to the use of a point-of-care ratiometric device based on intravascular readouts using fluorescently labeled dextrans. First, the real-time determination of GFR is based on a two-compartment model, thus eliminating the long time necessary for a one-compartment model that is based on elimination from the entire extracellular space.12-16 It is also more rapid than a continuous infusion approach that entails even longer time before a dynamic plasma equilibrium can be established between infusion and clearance.17-19 The ability of the rapid technique to quantify rapidly changing GFR has been previously reported for acute kidney injury in rats.7 Second, the safety of dextrans seems likely, as dextrans have been used clinically for many years in very large doses compared with the small amount used in this study.20-22 Third, dextrans can be correctly sized, highly conjugated with fluorescent molecules, and are very soluble in plasma, making them ideal markers.7 The extremely high sensitivity of the intravascular optical probe further reduces the amount of fluorescent injectate needed. The nearly continuous plasma data collection provides an unprecedented rich source of data for calculation of GFR. As we demonstrated recently, using two-photon microscopy in rats, the rapid sampling rate of data collection during the early time course is essential to help shorten the time needed for an accurate determination of GFR.7 This can be easily achieved with the fiber optical catheter and detection device. In addition, the larger fluorescent marker serves to rapidly quantify plasma volume, an important clinical parameter on its own, and eliminates the need for determining plasma concentrations of the smaller reporter molecule.7 Finally, the larger fluorescent marker, when used to develop the ratio, minimizes signal noise and detection abnormalities.7,9

The present technique has great utility for measuring GFR in many clinical situations. However, no situation is as important as acute kidney injury where a rapid diagnosis and quantification of the extent of injury are necessary. As both serum cystatin C and creatinine are slow to rise and not in equilibrium during the early phase of acute kidney injury, estimating equations are useless. Although structural biomarkers are being developed, the inability of these biomarkers to quantify the extent of injury will limit their effectiveness of clinical approaches to therapy.

The present approach does have limitations. First, an indwelling venous catheter is necessary for insertion of the optical probe and likely requires a vein size of 2.5 mm or larger. Recent studies in rats used a noninvasive detector of fluorescence to determine the GFR in rats.15,16 However, much longer time is required as this is dependent on extracellular detection and clearance of the small molecule used and not vascular clearance as performed in our studies using a two-compartment model. In addition, excessive amount of fluorescent marker is needed because of the loss of illumination and fluorescence emission in the tissue. For our studies, we have specifically designed the system so that a standard 18-gauge peripheral IV catheter will work. The study still takes about an hour, but we see no way to improve upon this. However, the data are obtained at the point-of-care, thus eliminating time-consuming laboratory procedures. Finally, motion artifacts due to animal movement have been a concern, but the ratiometric approach has greatly reduced this concern. In humans, we believe that there will be minimal spontaneous movement during the test, thus resulting in less optical noise from catheter movement.

The fluorescent approach presented here can be readily adapted for additional clinical studies. In addition, pharmacokinetic studies of fluorescently labeled molecules can also be accomplished rapidly with far richer data than allowed for by repeated blood sampling. Finally, development of specific fluorescent reporter molecules based on well-documented preclinical studies should allow for organ-selective evaluation using the approach delineated here.

**MATERIALS AND METHODS**

**Animal handling and data collection**

Protocols involving animals were approved by an institutional animal care and use committee and complied fully with recommendations outlined by the National Research Council and American Veterinary Medical Association.23,24 For the pig study, an adult 29.5 kg female Ossabaw miniature swine was anesthetized using 2.5-3.5% isoflurane, and the optical fiber was inserted through a central venous catheter placed in the external jugular vein. Following determination of background fluorescence, 5 ml of 350 mg iodine per ml iohexol (Omnipaque, GE Healthcare, Princeton, NJ) was injected as a bolus via a peripheral intravenous catheter, followed immediately by 5 ml of a fluorescent conjugate mixture containing 75 mg 5 kDa FITC-inulin (Sigma, St Louis, MO) and 75 mg 150 kDa Texas Red dextran (a gift from FAST, Indianapolis, IN). For the dog pilot study, peripheral 18-g, 1.2-inch intravenous catheters were placed in the left cephalic and left lateral saphenous veins of dogs. Following mild sedation with 0.1 mg/kg intravenously administered acepromazine or anesthetization with isoflurane, 0.9% NaCl equal to 2.5% of body weight was administered over 10 min; dogs with mild sedation were then minimally restrained in lateral recumbency for the entire procedure. The optical fiber was inserted through the cephalic catheter and, following determination of background fluorescence using the ratiometric analyzer, an intravenous bolus injection of 4 ml of 350 mg iodine per ml iohexol through the saphenous catheter was administered, immediately followed by a mixture of 69-175 mg 5 kDa fluorescein-dextran and 34-75 mg 150 kDa 2-sulfohexamidine-dextran dextran obtained from FAST.

EDTA-anticoagulated blood samples were collected immediately before and 30, 60, 90, 120, 180, 240, and 360 min, or immediately before and 5, 10, 20, 60, 180, 240, and 360 min following injections in the pig and dog, respectively. All blood samples were centrifuged within 3 h of collection and supernatants stored at −20°C until analysis. Fluorescent conjugate concentrations were determined using an M5 SpectraMax spectrophotometer (Molecular Devices, Sunnyvale, CA); iohexol concentrations were determined as previously reported.25,26 Data collection using the ratiometric device was begun ~1 min before the infusion of fluorescence conjugates, and continued for ~60 min. Sampling rate was 128 Hz at a rate of 1 point/s, and the data from each channel, as well as the green/red fluorescence ratio, were converted by the analyzer into smoothed real-time fluorescence curves, which were continuously displayed during data collection.

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Pharmacokinetic modeling and GFR determination

A two-compartment pharmacokinetic model, as described previously, was used for determination of GFR. Pig GFR was determined by the iothalamate method described previously, as well as the fiberoptic ratiometric method described below. In addition to these two techniques, dog GFR was also determined spectroscopically using the plasma concentrations of small fluorescent conjugate. Conventional approach to determine GFR requires determinations of the plasma concentrations of the GFR reporter from multiple blood samples drawn over 5–6 h. As was demonstrated recently, both requirements can be eliminated by using the ratiometric two-compartment model. We applied this approach, established using two-photon microscopy in rats, to the current studies in large animals using the optical fiber device described above. In brief, following bolus injection, the infused smaller dextran molecules are cleared by the kidneys or redistribute from the vascular into the interstitial space, but do not move intracellularly; the large dextran molecules are retained in the vascular space. The time course of the background-corrected fluorescence ratio of the freely kidney filterable reporter (5 kDa fluorescein-dextran or 5 kDa FITC-inulin) to the nonfilterable vascular marker (150 kDa Texas Red dextran or 5 kDa 2-sulfohexamine-rhodamine–dextran) was fitted to a bi-exponential equation (Equation 1) using the Sigma plot program to obtain the amplitudes and decay constants:

\[ R = Ae^{-\alpha t} + Be^{-\beta t} \]  

(1)

where \( R \) is the green/red fluorescence ratio, \( A \) and \( B \) are the initial amplitudes, and \( \alpha \) and \( \beta \) are the decay constants. GFR was then calculated using the equation:

\[ \text{GFR} = \frac{V}{\alpha + \beta} \]  

(2)

where the plasma volume \( V \) was determined via spectrophotometry (SpectraMax M5, Molecular Devices) by measuring the plasma concentration of the nonfilterable large dextran 10 min post intravenous injection, thus eliminating the need to quantify plasma concentration of the GFR reporter over time.

DISCLOSURE

Dr Molitoris receives grant funding from and is a founding member and the medical director of FAST.

ACKNOWLEDGMENTS

This work was made possible by funding from the National Institutes of Health grants P30-DK079312, R01-DK069408, and R41-DK079477 and from FAST Diagnostics, BioCrossroads, and the Indiana Economic Development Corporation’s 21st Century Fund.

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