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.

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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 m^2) 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[7] 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
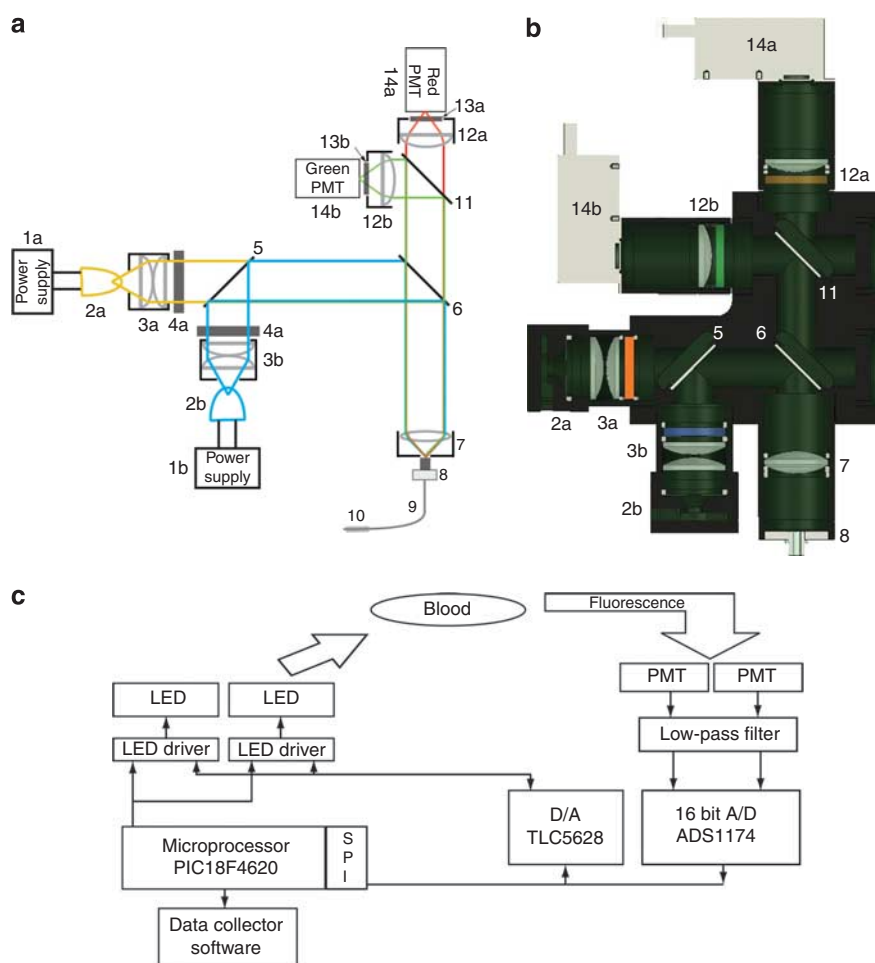

Figure 1 | **Design of the ratiometric optical fiber system.** (**a**) Optical layout. 1a and 1b, light-emitting diodes (LED) power supplies; 2a and 2b, amber and blue LEDs, respectively; 3a and 3b, collimating condensers with apertures; 4a and 4b, excitation bandpass filters; 5, excitation dichroic 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 12 b, 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.
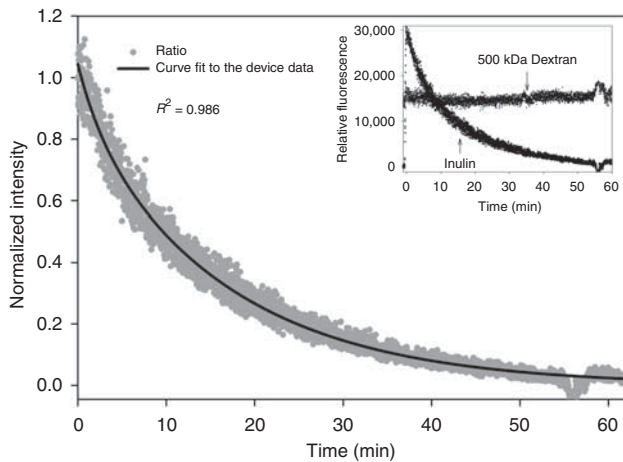
Figure 2 | Quantifying plasma volume and glomerular filtration rate in a pig using fluorescent inulin and dextran. Time courses for the reporter 5 kDa fluorescein isothiocyanate-inulin and the marker 150 kDa Texas Red dextran after a co-injection into an Osabaw swine. The fluorescence ratio (inulin/dextran) data (dots) were fitted to a bi-exponential equation and the fitted curve is shown as a solid line. The original data that generated the ratio are shown in inset.

a 1 mm aperture. The insertion of the fiber optic cable into the vein was facilitated by passing it through a standard angiocatheter. The length of the optical fiber was designed such that it extends ~1 cm beyond the distal tip of the intended intravenous catheter. The mixed fluorescence signal returned by the fiber passed through the same dual-band beam splitter that reflected the excitation wavelengths. The red and green emissions were separated by a Semrock FF560-Di01 dichroic mirror. The emission single bandpass filters for the green and red channels were a Semrock FF01 525/39 and a Chroma 640/50 m, respectively. A side-on Hamamatsu photo-multiplier tube (PMT) H9650-20 was used for both channels.

Electronic system

The electronic system of the device is shown in Figure 1c. The LEDs were driven by a voltage-controlled current source built around a MOSFET and operational amplifier. The control voltage was provided by the digital-to-analog converter (TLC5628, Texas Instruments) that communicated with the microprocessor (PIC18F4620, Microchip) via the serial peripheral interface port. The modulated LEDs provided 125 ms illumination pulses. Fluorescence emissions from the two conjugates were collected by two PMTs. The gain of the PMTs was controlled by the control voltage that was generated by another digital-to-analog converter controlled by the microprocessor. The analog signal from each PMT was filtered before feeding it into a 16-bit Delta-Sigma modulation analog-to-digital converter (ADS1174, Texas Instruments) operating at 128 samples per second. Accurate timing of the LED on/off time with synchronization of the A/D readings allowed data averaging and subtraction of ambient light and dark current errors, producing a final sampling rate of 1 Hz. The final data were transmitted via a USB port to a laptop computer.

Determination 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.[7] 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. 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.[8] 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.[7,9] 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.[7] This allowed for very small amounts of fluorescent dextrans to be used in our studies, and has
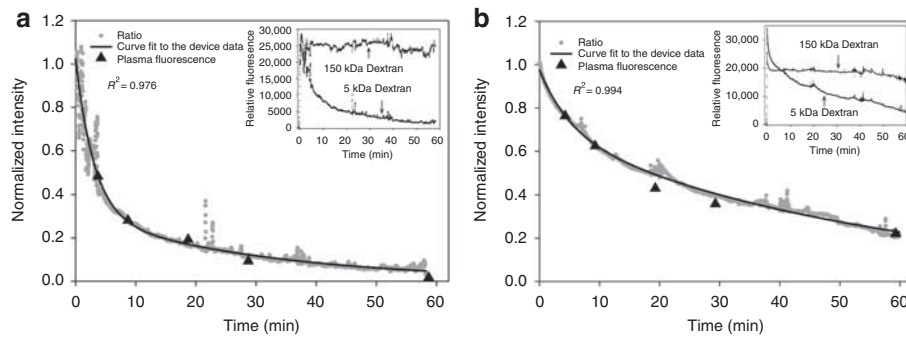
Figure 3 | Quantifying plasma volume and glomerular filtration rate in dogs using fluorescent dextrans. The data shown in **a** and **b** were generated from a dog under physiological conditions and a chronic kidney disease model, respectively. The 5/150 kDa ratio data (dots) from the optical device 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.

Table 1 | Normal and CKD GFR of dogs

Dog ID	GFR (ml/min/kg) by spectroscopy	GFR (ml/min/kg) by the device	GFR (ml/min/kg) by iohexol	Condition	Weight (kg)	Plasma volume (ml)
1	4.06	4.25	4.4	Normal	22.5	1344
2	3.76	4.8	4.5	Normal	33	1838
3	3.77	3.35	4.07	Normal	27.3	1352
4	1.27	1.19	1.45	CKD	29.8	1482

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.

important clinical safety and financial implications. In addition, the use of one fluorescent chimera as a plasma volume marker, and a second freely filterable small highly fluorescent reporter molecule to quantify the rate of plasma clearance as an assay for GFR, replaced the necessity of concentration measurements of the filtration reporter with relative fluorescence intensity determinations. Calibration of the detection device and plasma volume marker fluorescent dextran must still be accomplished. However, the relatively stable impermeable nature of this large marker molecule allows for accessing the plasma volume accurately. For the studies presented here, the use of the large dextran marker was in transition from a very large molecule (500 kDa) labeled with Texas Red in our rat studies[7] to a more clinically acceptable marker yet still with a size preventing movement into the extravascular space. We did not go smaller than 150 kDa to avoid movement of the marker molecule into the extravascular space, especially in disease states such as sepsis. We have also substituted a novel dye, 2-sulfohexamine-rhodamine-dextran, with reproducible production characteristics allowing for clinical use not possible with available rhodamine derivatives. We also converted to a freely filterable dextran, with the same molecular weight as that of inulin, for labeling with FITC instead of inulin for reasons previously explained.[7] We previously showed that the 5 kDa-FITC-dextran and 5 kDa FITC-inulin produced virtually identical decay curves and yielded the same GFR value in the same rat, indicating that there was no difference in the non-renal clearance between these two compounds. This contradicts the observation by Mehvar *et al.*[10] who reported

high non-renal clearance for a 4 kDa small dextran. If the non-renal clearance of our small dextran conjugate was indeed substantially higher than inulin, we would have obtained much higher GFR values. However, we have never seen this either with the fiberoptic device or spectroscopy approach, in which we used plasma samples for up to 6 hours.

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.[11] 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,[7] 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.

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 isofluorane, 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-sulfohexamine-rhodamine-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.

Pharmacokinetic modeling and GFR determination

A two-compartment pharmacokinetics model, as described previously,[7,26–29] was used for determination of GFR. Pig GFR was determined by the iohexol method described previously,[24,25] 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.[27] 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,[7] 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:

$$R = Ae^{-\alpha t} + Be^{-\beta t} \qquad (1)$$

where R is the green/red fluorescence ratio, A and B are the initial amplitudes, and α and β are the decay constants. GFR was then calculated using the equation:

$$\text{GFR} = \frac{V}{\frac{A}{A+B} + \frac{B}{A+B}} \qquad (2)$$

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.[7]

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