APPLICATION NOTE

GFR-Vivo 680 Fluorescent Imaging Agent



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An *in vivo* non-invasive method to determine glomerular filtration rate (GFR)

Abstract

Glomerular filtration rate (GFR) is the gold standard in kidney function assessment and is used to determine progression of kidney disease and drug-induced kidney toxicity. One of the most accepted ways to assess GFR is by measuring the rate of disappearance of labeled inulin from the blood; as inulin is completely filtered at the kidneys' glomeruli (but neither secreted nor reabsorbed by the tubules), this rate of disappearance is directly proportional to GFR. We have developed a near infrared (NIR) fluorescent-labeled form of inulin (GFR-Vivo[™] 680) in a spectral region providing low background and high tissue penetration (ex/em = 670/685 nm) for in vivo application. Fluorescence molecular tomographic (FMT) imaging of the heart was used to detect and quantify blood levels of GFR-Vivo 680 at multiple time points, providing the necessary data to calculate the clearance rates in individual animals. Following an intravenous bolus of NIR-Inulin in SKH-1E mice, FMT[®] images were acquired at 1, 5, 15, 30, and 45 minutes post-injection. Clearance rates were calculated using a two-compartment curve fitting, yielding average rates of 270 + 6 mL/min in normal mice. GFR-Vivo 680, in combination with FMT heart imaging, provides a non-invasive fluorescent imaging approach to generate consistent GFR measurements in models of kidney disease, dysfunction, and drug toxicity.



MATERIALS AND METHODS:

Fluorescent Agent

GFR-Vivo 680 is a near infrared (NIR)-labeled inulin molecule designed for determination of glomerular filtration rate through detection and quantification in the blood. The kinetics of non-invasive tomographic fluorescent imaging of heart fluorescence accurately represent the rate at which this agent is filtered through the kidneys' glomeruli.

| Basic Properties of GFR-Vivo 680 | | | | |
|----------------------------------|---------------------------|--|--|--|
| | GFR-Vivo 680 | | | |
| Agent Type | NIRF-labeled inulin | | | |
| Molecular Weight | ~6000 g mol ⁻¹ | | | |
| Absorbance | 670 nm | | | |
| Fluorescence emission | 685 nm | | | |

Agent Summary. Characteristics of the agent (MW/size, absorbance/emission) were determined in multiple independent studies

Models of kidney dysfunction

A variety of mouse models of kidney disease, dysfunction, or toxicity can show defined increases or decreases in glomerular filtration rate. These include uninephrectomy and kidney remnant models of chronic kidney disease (CKD), Type I (STZ-induced or spontaneous NOD mouse disease) and Type II (ob/ob mice) diabetic nephropathy, drug induced kidney toxicity with a variety of chemical agents, and infectious disease (e.g. Plasmodium berghei, Escherichia coli). In these studies, uninephrectomized and sham-surgery SKH-1E hairless mice (Charles River Laboratories, Wilmington, MA) were assessed seven days post-surgery as a model to validate GFR quantification.

In Vivo Heart Fluorescence Imaging

Heart fluorescence is only poorly detected by epifluorescence (2D) imaging, and rapid tomographic imaging is required for the purpose of quantifying levels of fluorescence in the heart. The FMT 2000 or FMT 4000 fluorescence imaging systems provide two (680 and 750 nm) or four NIR excitation lasers (635, 680, 750, and 790 nm), respectively, that are used for FMT imaging of small animals. A NIR laser transilluminates the animal (i.e. passes light through the body), with signal detection occurring via a thermoelectrically cooled charge-coupled device (CCD) camera placed on the opposite side of the imaged animal. Appropriate optical filters allow collection of both fluorescence and excitation datasets generated by raster-scanning the mouse, and the resulting imaging dataset is used to reconstruct fluorescence in 3D.

Protocol For GFR Assessment with GFR-Vivo 680

Study Preparation:

- 1. As GFR assessment requires carefully timed injections and imaging (1, 5, 15, 30, and 45 minutes) (Figure 1), it is essential to prepare ahead of time for optimal results. Group and number the mice to be injected and imaged.
- Mouse hair removal: Either genetically hairless mice (SKH-1E) or normal mice (BALB/c, C57BL/6, etc.), with depilation, must be used for optimal fluorescence tomographic imaging.
 - To minimize light scattering and absorption in heart imaging, hair is removed from the upper torso of all mice; depilatory cream (Nair lotion, Church and Dwight Co., Inc., Princeton, NJ) is applied thickly on skin over the upper torso (front, back, and sides) of each mouse, rinsed off with warm water, and reapplied until all hair has been removed.
 - Care should be taken to remove hair from an area larger than just the upper chest (i.e. including removal from mid-torso and neck regions, as well).
- 3. Establish the readiness of the FMT imaging system by checking the anesthesia chamber and connections to the FMT. Activate the anesthesia, setting evaporator to 3.5.
- 4. TrueQuant[™] software: Open FMT TrueQuant software and enter all necessary study information as a new study. Open the image acquisition software panel in readiness for the first mouse and select the first subject in the "Select Subject" field.
- Make sure you know ahead of time the proper positioning of the mouse in the imaging cassette to permit tomographic imaging of the thoracic region.



Figure 1. Kinetic Heart Imaging. Hairless or depilated mice can be imaged repeatedly over time to quantify levels of fluorescence in the heart as a surrogate for blood measurement.

Injection of Mice with GFR-Vivo 680:

- 1. Prepare the GFR-Vivo 680 agent according to included instructions.
- 2. Place a heating pad beneath the anesthesia induction chamber to keep the body temperature of the mouse constant. Be careful not to overheat. Anesthetize the first mouse by placing it in the gas anesthesia induction chamber.
- Prepare a notebook for entry of injection times. Use the time shown on the computer monitor of the imaging system to assure alignment of injection time with imaging times.
- Remove the mouse from the induction chamber when it appears completely anesthetized, and confirm the depth of anesthesia through unresponsiveness to toe pinch.

- 5. Inject 100 μ L (2 nmol) of GFR-Vivo 680 via the retro-orbital plexus (or tail vein) of the anesthetized mouse. Record the time as "injection time" or t=0.
- 6. Quickly position the injected mouse in the FMT's imaging cassette (supine) and position the top plate of the cassette. Gently adjust the cassette depth for proper and gentle restraint of the mouse (generally 13 mm depth for a 25 g mouse). This will not affect normal respiration but will secure the mouse in place. Steps 5 and 6 should take no longer than 20-30 seconds).

FMT 4000 heart imaging:

- Insert the mouse/cassette into the FMT imaging system and close the door. The correct subject to be scanned should already have been selected in the "Select Subject" field.
- Acquire the reflectance (2D) image of the mouse and quickly size and align the scan field to allow 45-50 scan points positioned to encompass the ribcage region (thorax) of the mouse (see Figure 2). Select "Add to Reconstruction Queue" for automatic background reconstruction of tomographic datasets.
- 3. Start the FMT acquisition scan to acquire the first time point scan, initiated as close as possible to 1 minute post-injection. Record the time that the scan is initiated. The scan will take 2-2.5 minutes.
- 4. As soon as the first scan is completed, turn down the evaporator to maintenance levels (set to 1). With the lower anesthesia setting, each mouse can be kept within the imaging chamber for the duration of the experiment.
- 5. At 4 or 5 minutes post-injection, initiate the second thoracic scan. Record the time that the scan is initiated.
- 6. Repeat at 15, 30, and 45 minutes. In some cases, you may want to extend the imaging to 60 minutes.
- 7. Note that scan initiation time can also be confirmed in the experiment tab for each animal scan (Created column).



Figure 2. TrueQuant Scan Acquisition. Following the acquisition of the reflectance image, a white light view of the mouse will appear with a default scan field superimposed on the image. Resize the scan field using the computer mouse to achieve full scan coverage of the upper chest in roughly a 7×7 grid, or larger.

Note: <u>Alternative overlap imaging of two mice</u>: for maximal imaging throughput, it is possible to image an additional mouse during the longer imaging intervals.

- a. Inject the second anesthetized mouse (described above) as the 5 minute scan of the first mouse is completed. Record the time for mouse 2, and replace mouse 1 in the cassette with mouse 2.
- b. Complete 1 and 5 minute imaging time points for mouse 2, and then switch back to mouse 1 for the 15 min time point for mouse 1.
- c. Alternate the mice to complete the imaging time points for both mice (t = 1, 5, 15, 30 and 45 min)

FMT 4000 analysis:

Proper quantitative analysis of heart fluorescence by the FMT System's TrueQuant software requires accurate and consistent positioning of 3D ROIs. Figure 3 shows an image of a mouse acquired on the FMT at the first time point, with an outline to indicate the ribcage. It is important to capture heart signal without capturing nearby liver signal (due to high vascularity of this organ). An ellipsoidal ROI is deliberately oversized to maximize capture of all of the heart's fluorescence. Viewed from the front, it is positioned roughly in the center of the ribcage, with care taken to avoid the top of the liver (the tip of which is behind the xiphoid cartilage). All of the time point data from a single mouse can then be opened within TrueQuant in the Analysis tab (set to 6 panels), and the ROI from the first time point can be copied to all of the subsequent time points.



Figure 3. TrueQuant ROI Placement. A 3D ellipsoidal ROI should be placed that is 9x11x6 voxels (width x height x depth) and localized centrally in the rib cage region and set to capture the first 6 depth voxels. Other signal will be seen due to circulation through blood vessels in the surrounding tissues.

No thresholding should be applied to the heart ROIs, and the pmol data will be used for subsequent calculations of blood concentration. This is repeated for each individual, image ultimately yielding quantification of the complete kinetic tomographic datasets for every animal. The complete pmol dataset is then exported to Microsoft® Excel® for further calculations.

Calculation of blood concentration:

Although the TrueQuant software calculates the nM concentration of fluorescence within the heart ROIs, this is an innacurate measure of blood fluorescence, because 1) it doesn't account for the volume of tissue versus blood in the ROI, and 2) the resolution of fluorescence tomographic reconstruction generally oversizes the volume of fluorescence due to the inherent properties of light propagation through tissue. This means that the pmol data are the most accurate readout (not subject to these artifacts), but they need to be converted to nM by dividing them by the blood volume within the heart.

A 25 g mouse has a blood volume of approximately 1.5 mL (see Table I). Heart fluorescence pmol are converted to nM by making the assumption that the heart contributes 7% of the blood volume, or 0.1 mL. Based upon these reported physiologic parameters of the mouse, the t=0 fluorescence concentration after injection of 2 nmol of GFR-Vivo 680 would be 1333 nM. All imaging time point values are determined by dividing the pmol value by 0.1 mL, yielding the concentration within the blood of the heart in nM.

| Table I. Vascular physiologic parameters in the mouse | | | |
|---|--------------------------|--|--|
| Physiologic Parameter | Measurement in 25g Mouse | | |
| Total blood volume | 1.5 mL | | |
| Heart blood volume | 7% of total blood volume | | |
| | = 0.1 mL | | |

The imaging times are noted during the *in vivo* study data acquisition, however the times should be adjusted to provide a more accurate estimate of time. As each tomographic imaging session for a 45-50 point upper torso scan takes approximately 2 minutes, we established each time point as the scan start time + 1 minute (the temporal midpoint of each scan). So, scan initiations at 1, 5, 15, 30, and 45 minutes yield actual data time points of 2, 5, 16, 31, and 46 minutes (see Table II). This modification of times has some impact on GFR calculation but will mostly affect calculation of blood half-life.

| Table II. Calculated FMT imaging times | | | | |
|--|------------------|-----------------------------|--|--|
| Imaging Start Time (min) | Scan Duration | Imaging Time Point (min) | | |
| 0 | N/A | 0 | | |
| 1 | 2 min | 2 | | |
| 5 | 2 min | 6 | | |
| 15 | 2 min | 16 | | |
| 30 | 2 min | 31 | | |
| 45 | 2 min | 46 | | |



Figure 4. Kinetic heart imaging by FMT. Two representative mice (Sham and Uninephrectomized) are shown at the indicated imaging times. Heart (only) signal in 3D is shown for each time point, with higher concentration signal represented by shades of red.

Calculation of glomerular filtration rate (GFR):

A two-compartment clearance model is used to calculate GFR, providing a good fit to both the early, rapid decay phase as well as the later, slower elimination phase. A variety of Pharmacokinetic software (including WinNonLin [Pharsight, Sunnyvale CA] and PK Solutions [SummitPK, Eugene OR]) can be used to calculate pharmacokinetic parameters such as blood half-life and clearance rates. Alternatively, GFR can also be calculated on GraphPad Prism (GraphPad Software, San Diego, CA) using the equation:

GFR = I / (A/a + B/b)

I is the amount of inulin delivered in bolus injection; A and a are the y-intercept and decay constant of the initial rapid decay phase, respectively; and B and b are the y-intercept and decay constant of the slow elimination phase, respectively (Qi et al., 2004).

For our GFR studies, we used a freely available, menu-driven add-in program for Microsoft[®] Excel[®] written in Visual Basic for Applications (PKSolver, Zhang Yon, China Pharmaceutical University). A detailed description and comparison by Zhang *et al*, can be found in the journal Computer Methods and Programs in Biomedicine 99 (3); 306-314 (2010). All data were tabulated in Microsoft[®] Excel[®], and the Two Compartment IV Bolus fit of the PKSolver Add-In was used to generate curve fits and calculations of the clearance rates for each mouse in uL/min.

To test the imaging methodology and analysis techniques of non-invasive GFR quantification, we imaged the effect of the removal of one kidney (uninephrectomy) on GFR-Vivo 680 heart signal (Figure 4). Uninephrectomy should yield a delay in the clearance of circulating fluorescence (assessed in the heart) which should translate into a 50% decrease in GFR as compared to sham surgery mice. Times were recorded for each Sham and Uninephrectomized mouse, and the time points were defined relative to injection time (t 0), taking into account the 2 minute scan time by adding 1 minute (mid-point of each scan) to each imaging time. In Table III, data from a representative Sham surgery mouse (A) and a representative Uninephrectomized mouse (B) are shown. The total pmols of fluorescent signal from GFR-Vivo 680 were converted to blood concentration (nM) by dividing by 0.1 mL (heart blood volume) as described in the previous section and in Table I. Within Excel, this dataset was analyzed using the PKSolver Add-in with a 2-compartment curve fitting optimized for IV bolus (Figure 5). Briefly, for each mouse the Time and Concentration datasets are selected, time unit is set in "min", concentration in "nmol/L", Dose at "2", and Dose Unit as "nmol" (Figure 6).

Curve fitting for these two representative animals fit the observed data well and revealed a delay in apparent clearance of GFR-Vivo 680 from circulation (Figure 7). GFR for Sham and Uninephrectomized mice were determined to be 251 and 120 µL/min, respectively (Table IV), in agreement at the level of both established normal GFR values from the literature and with regard to the quantitative effect of uninephrectomy. Other calculated pharmacologic parameters, including alpha- and beta half-lives, exposure levels (AUC and AUMC), mean residence time (MRT), and steady-state volume of distribution (Vss) all show the expected differences between these two mice.

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| 1 | .7 | | | | 2:19 | 2 | | 76.9 | 769 | | |

Figure 5. Curve Fit Selection with PKSolver. The PKSolver add-in, opened within Microsoft Excel, offers many options for curve fitting of pharmacokinetic data. The 2-compartment IV bolus curve fit was used to best capture the typical kinetics of inulin clearance.

Table III. Converting FMT pmol to blood concentration

| A. Shan | n Mouse | | | |
|------------------|---------------|--------------------|------------------|--|
| Ti | mes | Heart Measurements | | |
| Recorded Time | Time point | Measured pmol | Calculated nM | |
| 2:10 | 0 | - | 1333 | |
| 2:11 | 2 | 63.5 | 635 | |
| 2:15 | 6 | 40.4 | 404 | |
| 2:26 | 17 | 12.7 | 127 | |
| 2:41 | 32 | 4.9 | 49 | |
| 2:55 | 46 | 3.1 | 31 | |

B. Uninephrectomized Mouse

| | • | | |
|------------------|---------------|--------------------|------------------|
| Times | | Heart Measurements | |
| Recorded Time | Time point | Measured pmol | Calculated nM |
| 2:18 | 0 | - | 1333 |
| 2:19 | 2 | 76.9 | 769 |
| 2:22 | 5 | 58.1 | 581 |
| 2:32 | 15 | 29.4 | 294 |
| 2:47 | 30 | 13.7 | 137 |
| 3:03 | 46 | 17.9 | 179 |







Figure 7. Kinetic Blood Clearance Curves. Kinetic heart datasets from representative Sham and Uninephrectomy mice are shown.

In vivo GFR assay performance metrics:

Averages for normal mice and uninephrectomized mice were compared across multiple studies. GFR-Vivo 680 assessment of GFR by FMT tomographic imaging of the heart compared favorably with regard to normal animals, yielding averages of 274 +/- 56 μ L/ min in five studies. Uninephrectomized mice showed an average GFR value of 132 +/- 12 μ L/min in three studies.

Power analysis studies (modeled on actual variability of empirical data) were performed to predict the numbers of animals needed per group to detect GFR decreases of 30-50% (to approximate typical findings from toxicity and nephrectomy studies), and 30% to 100% increases (to approximate expected increases in GFR expected in diabetes). Our approximations indicated that as few as 4-5 mice per group were required to detect 2-fold changes (either 2-fold increase or 50% decrease) and 10-15 mice were required to detect 30% changes relative to controls. A typical blood collection study (*ex vivo* assessment of insulin levels) could require from 30-60 mice, depending on institutional IACUC rules regarding repeat blood sampling.

SUMMARY

The present studies provide evidence for the utility of GFR-Vivo 680, in combination with FMT imaging, in the non-invasive guantification of glomerular filtration rate (GFR). Imaging and analysis techniques are described, and the data generated in normal and uninephrectomized mice show clear data in agreement with the range of published GFR values. Further, GFR-Vivo 680 assessment of GFR yields an expected 50% decrease upon removal of one kidney. Inter-animal variability was 15-20%, mostly attributed to the variability of the biology itself from animal-to-animal. In addition, differences would be expected in comparison of male to female mice, mice of different ages, and in profiling different mouse strains. Looking at 5 independent studies in SKH-1E mice, inter-assay variability within the same mouse strain was < 10%, indicating that the average GFR of small groups of mice was fairly consistent despite the animal-to-animal variability.

Statistically significant changes in GFR upon uninephrectomy were easily detected with only 4-5 mice per group (imaged at multiple time points), and our extrapolations predict that as few as 10-15 mice per group would be required to detect a 30% changes in GFR. Even increases in GFR up to 3-fold (i.e. more rapid clearance from the blood should be readily detectable using the protocol described. In conclusion, heart tomography of GFR-Vivo 680 fluorescence should provide a robust approach to generate consistent GFR measurements in models of kidney disease, dysfunction, and drug toxicity.

Table IV. GFR and other PK measures established from FMT heart measurement of Sham and Uninephrectomy mice

| Parameter | Unit | Sham | Uninephrectomy |
|-----------|--------------|-------|----------------|
| t1/2Alpha | min | 0.5 | 1.3 |
| t1/2Beta | min | 7.3 | 17.9 |
| CL | μL/min | 251 | 120 |
| AUC 0-t | nmol/L*min | 7860 | 14039 |
| AUMC | nmol/L*min^2 | 78975 | 398113 |
| MRT | min | 9.9 | 23.9 |
| Vss | mL | 2.5 | 2.9 |

Table V. Non-invasive GFR-Vivo 680 assay performance metrics

| Result | Comment |
|---------------------------------------|--------------------------------|
| Average GFR in 25g female SKH-1E mice | $274 \pm 56 \ \mu\text{L/min}$ |
| (n = 11) | |
| Average GFR in uninephrectomized 25 g | $132 \pm 12 \ \mu$ L/min |
| female SKH1-E mice (n = 11) | |
| Intra-assay variability | 15-20% |
| (Coefficient of Variation) | |
| Inter-assay variability | < 10% |
| (Coefficient of Variation) | |
| n required to detect 30% change | 10-15 mice per group |
| n required to detect 2-fold change | 4-5 mice per group |

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