**Responses to Editorial Comments and Reviewers:**

Responses to Editorial comments:

1) Insertion details for the catheter in step 2.3 are missing.

**Response: Insertion details have been included.**

2)References need to be in JoVE format.

**Response: References are in JoVE format.**

3)Please note that reviewers 1 and 2 have raised some serious concerns about aspects of your manuscript. Please thoroughly address or rebut each individual comment below to further strengthen and clarify your submission. • Please note that we do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

**Response: Responses to reviewers are below.**

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Responses to Reviewers' comments:

Reviewer #1:

Overall Review.

The likelihood that this could be used as a hands-on teaching laboratory is low. It is not clear to what level of students this physiology lab demonstration would be useful. A simple error in blood collection may result in the loss of a large volume of blood in the rat and would terminate the experiment. Additional details are required to test for the adequacy of depth of anesthesia and maintenance of body temperature. The usefulness of this teaching method for a lab demonstration is also limited due to the small field of view for students to observe the cannulation of femoral vessels and to wait 1-2 hours for the equilibration period. The addition of a teaching dissection microscope would be advantageous. Small groups of 3-4 students might be able to observe a skilled rodent surgeon perform the studies. The students might perform the analytical measures with supervision by technical staff. Students could collect, tabulate, and calculate all of the renal parameters and write a lab report based on the outcome of the study. The usefulness of the methodology for experimental scientific research is limited by the paucity of information on criteria for acceptable data.

**The authors thank the reviewer for the thorough review of the paper. We understand that there are limitations regarding the effectiveness of the demonstration to teach all aspects of conducting a renal function experiment. The description of this procedure as a “teaching demonstration” is therefore inaccurate. We have redefined the procedure as a “lab demonstration”. We have changed the phrase “hands-on teaching laboratory” to “demonstration laboratory”. The purpose of the demonstration is to provide students with an overall conceptual understanding of how to measure renal function. We have revised the manuscript to convey this purpose and to provide a more detailed description of procedures such assessing depth of anesthesia, maintenance of body temperature, femoral vessel cannulation, and sample collections.**

Minor

The authors might add a citation of the first discovery of inulin as a marker of glomerular filtration.

The authors might cite their 2012 JoVE publication which contains a detailed description of catheter implantation in the anesthetized rat.

**These references (ref # 5 and #6) have been added to the revised paper.**

Graduate students with small animal surgical skills may be the only students that could assist in the administration of fluids and collection of blood samples in an anesthetized rat. Research technicians with small animal surgical skills may be able to initiate these studies in the laboratory. Important details of maintaining adequate blood pressure and plasma volume during an acute experiment are not presented in adequate detail to provide usefulness for laboratories not familiar in the study of renal function in anesthetized rats.

**A paragraph describing the roles of assistants in the demonstration has been added to the Discussion.**

Abstract.

'evaluating renal effects of disease' should read the effects of disease on renal function.

**We have revised the statement in the abstract.**

If the overall goal of this paper is to serve as a 'hands-on teaching laboratory' then this might be reflected in the title.

**We have deleted the phrase “hands-on teaching laboratory” from the revised paper. The title reflects that the activity is a “demonstration laboratory”.**

Introduction.

The kidneys regulation extracellular fluid water and electrolyte content. The kidneys do not regulate the balance of intake and output of water. The kidneys maintain extracellular fluid water and electrolyte content in the face of changes in the intake and output of fluids and electrolytes. Urine is not excreted to the bladder. Urine is temporarily stored in the bladder and excreted from the body by relaxation of the external urethral sphincter. The basic properties of inulin should be presented.

**The introduction has been revised.**

Line 80-81.

Active participation of students in the execution of the experiment may be limited to the tabulation and calculation of the data collected by an experienced technician or student.

**A paragraph describing the roles of assistants in the demonstration has been added to the Discussion.**

Protocol.

1.2) add abbreviation for BSA **The abbreviation has been added.**

2.1) the order of events is incorrect, record BW and then induce anesthesia, add air flow rate

**The order is correct. The animal is weighed after the induction of anesthesia.**

2.3) add size and type of catheter and the degree of tapering and sutures

**In an effort to stay within the space constraints for the paper, we have referred the reader to the JoVE video (ref #6) for this technique.**

2.5) bladder catheter is heat flared **This description has been revised.**

3.1) the time to perform the surgery and then to wait for a 1-2 hour equilibration is not conducive to teaching a laboratory exercise for students, the students might arrive to the laboratory at the conclusion of the equilibration period

**This has been added to the Discussion of the revised paper.**

3.2) is a urine flow rate of 20 microL/min adequate for a rat of a given body weight

**Yes, 20 microL/min is adequate urine flow for the demonstration as it provides a minimum of 200 microliters of urine sample for each 10 minute collection period.**

3.3.1) volume of heparinized saline used to flush the arterial line should be provided, catheter should be as short as possible to limit the volume of saline flush

**This has been added to the revised paper.**

3.4) dose of furosemide should be based on kg of body weight, instructions for ensuring that air is not injected into the arterial line are necessary

**This has been added to the revised paper.**

3.5.1) It is not clear why the urine samples are collected during 10 min and post-drug samples are made at 5, 10, 15 min after furosemide.

**All urine samples were collected during a 10 min collection period. The 3 Post Drug Samples were collected at various times points after furosemide in an effort to capture the time course of the drug’s effects on renal function.**

3.5) details should be included to decapsulate and blot kidneys prior to weighing.

**This has been added to the revised paper.**

5.1.3) Filtered load of sodium is the more common terminology.

The manuscript must be very carefully reviewed for the proper use of the Greek symbol for micro. There are many instances in the text and tables in which the lowercase letter 'u' is used incorrectly.

**The corrections have been made in the revised paper.**

RESULTS.

GFR is the rate by which plasma (not blood) is filtered by the kidneys.

**The corrections have been made in the revised paper.**

Line 188. Greater amounts of Ca, K, and water compared to what? Pre-drug levels? Comments regarding significance of the increase in urine flow rate are inappropriate since statistically significant results are not included in the paper.

**The corrections have been made in the revised paper.**

Line 193. Include g per kidney weight, what is the importance of calculating GFR based on kidney weight?

**The kidney weight is shown in Table 2. We have included the calculation of GFR based on kidney weight as this calculation is often used to describe GFR in animal studies.**

Line 205. A blank sample should be included in the standard curve.

**The values for the reagent blank were added to the standard curve presented in Table 1.**

Line 225. urine sodium, potassium and inulin concentrations.

**The corrections have been made in the revised paper.**

Discussion

Line 245. To who does the lack of risk of radiation are the authors referring?

**The corrections have been made in the revised paper.**

Table 1. request inclusion of plasma inulin

**Due to the page limits of the paper, we have included one representative inulin standard curve for urine samples.**

Table 2. mean arterial pressure, duration of sample collection, plasma electrolytes units of mEq/L which is the usual units for human

**“BP” has been changed to “MAP”; “sample time” has been changed “duration of sample collection” in Table 2.**

Table 3. urine flow rate should be recorded in microL; the GFR of the pre-drug 1 and 2 varies by 30% and does not represent a stable experimental animal

**We agree that the degree of variability in GFR between the 2 pre-drug samples may indicate an unstable experimental animal, but for the purpose of the demonstration some variation in the baseline GFR can be tolerated. We have included a statement in the discussion that addresses the importance of steady urine flow rates at baseline.**

List of Materials - recommend alphabetical order; FITC-inulin instead of Inulin-FITC; List of Equipment - recommend alphabetical order

**The list has been organized in alphabetical order.**

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Reviewer #2:

Manuscript Summary:

A nicely detailed description of a standard GFR measurement in a rat is provided, using FITC-inulin as the filtration marker.

Major Concerns:

1) This is a standard technique used to measure GFR in rats. It is not substantively different that that used in any number of labs across the country. I was expecting that there would be some technique or "trick" that would make the protocol more expedient for use in a student lab - but there is not.

**The authors thank the reviewer for the thorough review of the paper. The authors’ intention was to provide a description of how to provide a lab demonstration for students to understand the basic experiment by which to measure renal function by traditional methods. We have revised the manuscript to convey this purpose and to provide a more detailed description of how the demonstration can be accomplished in an expedient manner while allowing the students to participate in sample collection and calculations of renal function.**

2) It is not reasonable to expect students (undergraduate or graduate) with little or no previous surgical experience to successfully accomplish a cannulation of the femoral vessels. Even in adept students, and with personal instruction, this takes a fair amount of practice to perform successfully. Likewise, management of surgical anesthesia using isoflurane requires some amount of experience and expertise - especially in a procedure as lengthy as this one.

**The description of this procedure as a “teaching demonstration” is inaccurate. We have redefined the procedure as a “lab demonstration”. The revised paper describes the role of the students in the lab demonstration more precisely. Specifically, the pre-lab preparation of FITC-inulin solution, and surgical preparation of the animals are performed by experienced technicians prior to the start of the demonstration. The students arrive for the demonstration at the end of the 1-2 hour inulin equilibration period and are instructed on how to collect blood and urine samples.**

3) The inulin standard curve, as shown and described, is problematic. All existing literature indicates that it should be a linear relationship. In our own experience this has always been the case; out of curiosity, we performed a standard curve in EXACTLY the same fashion as described in this paper, and the resulting relationship that we recorded was linear. Furthermore, the data in figure 1 is graphed incorrectly, since the x-axis is not a linear scale (it is categorical); however when graphed correctly, the relationship is still inexplicably non-linear (flattening at higher concentrations). Even still, if one were to concede to a "4-parameter logistic function" - a description of how the student might accomplish this complex piece of math is not provided.

**The data in figure 1 was plotted with a log scale, not categorical, in order to show the values on the x axis with equal spacing . However, in retrospect, we agree that this was confusing. Therefore we have graphed this data with a linear scale for the x axis and are also now able to include the values for the blank or zero point. We also expected a linear relationship when we initially performed this lab demonstration but found that the standard curve was not linear for the instrument that we have used. Many plate readers or scientific statistical /graphing software packages include the ability to do nonlinear regression analysis such as the 4-parameter logistic function since this function is usually used for immunoassay analyses. The software package we used is Sigmaplot.**

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Reviewer #4:

Manuscript Summary:

Hinojosa-Laborde and coauthors describe renal function assays in a rat. This is a well-written manuscript with the appropriate methodology. I have a few suggestions:

Major Concerns:

1. The authors used FOTC-labeled inulin and fluorescent plate reader to measure inulin concentrations in blood and urine. Even though this is an excellent method of detection, the equipment is not a standard one and may be costly. Alternative methods, such as the anthrone method, should be at least discussed and described as well.

**The authors thank the reviewer for the thorough review of the paper. The discussion of the traditional inulin measurement based on the anthrone method is** **has been expanded in the Discussion.**

2. The authors used an inhalation anesthesia by isoflurane, which also requires special equipment. Alternative methods, such as inactin, should be described.

**A discussion of alternative anesthetic agents has been expanded in the Discussion.**

3. The authors did not specify the weight of the animals used, the inulin infusion rate is provided in ml/h, whereas it should be in ml/kg/h.

**The body weight range appropriate for the lab demonstration has been included in Section 2.1. The inulin infusion has been expressed as ml /hr/100 g in Section 3.1.**

4. The femoral artery caterer should be flashed with heparin-containing saline solution after each blood collection to prevent thrombosis.

**This has been added to the revised paper (Section 3.3)**.

5. The final concentrations of inulin/albumin should be provided in addition to the preparation protocol.

**This has been added to the revised paper (Sections 1.2 and 1.3)**.

6. Blood centrifugation time is not provided, and 18,000G appears to be too high, 1200G for 5 min is usually sufficient to separate blood cells from plasma.

**We apologize for the typographical error. The correct centrifugation rate is 1800 G is provided in the revised paper (Section 4.2).**

Minor Concerns:

7. The short abstract should mention that this is method in rats.

**This has been added to the revised paper.**

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Reviewer #5:

Manuscript Summary:

Dr. Hinojosa-Laborde and coworkers describe renal function measurement in the rat using constant FITC-inulin infusion.

Major Concerns:

1) Title: The title should include "GFR" to accurately reflect the presented protocol

**The authors thank the reviewer for the thorough review of the paper. The title of the protocol has been revised.**

2) I am wondering whether auto-fluorescence is present, especially in the undiluted plasma, and suggest that a plasma sample is assayed for FITC prior to infusion.

**As a result of the reviewer’s question we measured blank values with and without plasma in the sample and found that plasma actually slightly decreased the fluorescence of the blank. Thus we found no evidence for auto-fluorescence. However, we agree that blank plasma and urine samples should be collected and used to correct the fluoresence of the samples. The values for the reagent blank were added to the standard curve presented in Table 1.**

Minor Concerns:

line 53 - please be more specific regarding specialized equipment required for alternative methods

**The discussion of alternative methods has been added to the Discussion section.**

line 60 - the statement is not supported by evidence

**We have provided a reference to support the statement.**

line 76-78 - measurement of GFR is not required for calculation of FE of Na and K, because urine flow rate cancels-out

**The correction has been made in the revised paper.**

line 121 and elsewhere - can the blood volume not be reduced, to avoid repeated large-volume sampling? I am concerned that renal blood flow may drop during the experiment

**The blood sample size is dependent on the equipment available for analysis. The sample volume required for the equipment we used was 0.5 ml.**

line 138 - the spinning force (18,000g) seems overly excessive - cells may lyse and compromise the fluorometric reading

**We apologize for the typographical error. The correct centrifugation rate is 1800 G is provided in the revised paper (Section 4.2).**

line 162 and elsewhere - please consistently use the µ symbol and not u when denoting micro.

**The correction has been made in the revised paper.**

line 162 and elsewhere - µM (micromolar) are not the correct units - should be µmol (micromol)

**The correction has been made in the revised paper.**

line 193 - why is the GFR presented as ml/min/g? correction for renal mass is irrelevant for the successive calculations

**We have included the calculation of GFR based on kidney weight as this calculation is often used to describe GFR in animal studies.**

lines 197-199 - furosemide injection would typically cause vasodilation and reduced BP. also, increased BP does not explain the increase in GFR

**The description of the renal effects of furosemide has been revised.**