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Physiology Lab Demonstration: Glomerular Filtration Rate in a Rat

--Manuscript Draft--

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Abstract:	Measurements of glomerular filtration rate (GFR), and the fractional excretion of sodium (Na) and potassium (K) are critical in assessing renal function in health and disease. GFR is measured as the steady state renal clearance of inulin which is filtered at the glomerulus, but not secreted or reabsorbed along the nephron. The fractional excretion of Na and K can be determined from the concentration of Na and K in plasma and urine. The renal clearance of inulin can be demonstrated in an anesthetized animal which has catheters in the femoral artery, femoral vein and bladder. The equipment and supplies used for this procedure are those commonly available in a research core facility, and thus makes this procedure a practical means for measuring renal function. The purpose of this video is to demonstrate the procedures required to perform a lab demonstration in which renal function is assessed before and after a diuretic drug. The presented technique can be utilized to assess renal function in rat models of renal disease.
Author Comments:	Thank you for the opportunity to submit a manuscript of a renal function lab demonstration to the Journal of Visualized Experiments. This laboratory demonstration is a part of the "Short Course in Integrative and Organ Systems Pharmacology" offered by the Michigan State University, East Lansing, MI. This course, under the direction of Dr. Peter Cobbett, has successfully provided students from various educational backgrounds a hands-on laboratory experience in small animal testing methods. The purpose of the various lab demonstrations is to expose students to techniques typically used in assess various physiological functions in small animals. Our work specifically addresses the hands-on method for teaching the techniques for measuring renal function in the rat.
Additional Information:	
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If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

June 20, 2014

Dear JoVE editorial staff,

Thank you for the opportunity to submit a manuscript of a renal function lab demonstration to the Journal of Visualized Experiments. This laboratory demonstration is a part of the "Short Course in Integrative and Organ Systems Pharmacology" offered by the Michigan State University, East Lansing, MI. This course, under the direction of Dr. Peter Cobbett, has successfully provided students from various educational backgrounds a hands-on laboratory experience in small animal testing methods. The purpose of the various lab demonstrations is to expose students to techniques typically used in assess various physiological functions in small animals. Our work specifically addresses the hands-on method for teaching the techniques for measuring renal function in the rat.

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KEYWORDS:
kidney; rat; glomerular filtration rate; urine flow rate; sodium excretion; potassium excretion; filtered load; blood pressure

SHORT ABSTRACT:
The purpose of this protocol is to demonstrate the principles and techniques for measuring and calculating glomerular filtration rate, urine flow rate, and excretion of sodium and potassium in a rat. This demonstration can be used to provide students with an overall conceptual understanding of how to measure renal function.

LONG ABSTRACT:
Measurements of glomerular filtration rate (GFR), and the fractional excretion of sodium (Na) and potassium (K) are critical in assessing renal function in health and disease. GFR is measured as the steady state renal clearance of inulin which is filtered at the glomerulus, but not secreted or reabsorbed along the nephron. The fractional excretion of Na and K can be determined from the concentration of Na and K in plasma and urine. The renal clearance of inulin can be demonstrated in an anesthetized animal which has catheters in the femoral artery, femoral vein and bladder. The equipment and supplies used for this procedure are those commonly available in a research core facility, and thus makes this procedure a practical means for measuring renal

function. The purpose of this video is to demonstrate the procedures required to perform a lab demonstration in which renal function is assessed before and after a diuretic drug. The presented technique can be utilized to assess renal function in rat models of renal disease.

INTRODUCTION:

The most important function of the kidney is the homeostatic regulation of extracellular water and electrolyte content. The kidneys closely regulate extracellular water, sodium (Na) and potassium (K) to maintain normal physiological levels. Disturbances in renal function can result in serious metabolic disorders which can be fatal. The basic renal process occurs in the nephron and begins with the filtration of plasma at the glomerulus and ends with the excretion of urine. Other processes that determine the final concentration of water, Na and K in the urine are secretion and reabsorption within the nephron. Measurements of glomerular filtration rate (GFR) and the fractional excretion of Na and K are critical in assessing renal function in health and disease. The reader is referred to previously published review articles and textbooks for a more thorough discussion of kidney function[1-4].

GFR can be measured as the steady state renal clearance of inulin which is filtered at the glomerulus, but not secreted or reabsorbed along the nephron[5]. While this technique requires anesthesia, surgical preparation, and a terminal experiment, it is considered the gold standard of GFR measurement. Using inulin that is tagged with fluorescein-isothiocyanate (FITC), plasma and urine concentration of FITC-inulin can be easily measured in small volumes and used to calculate GFR during multiple time points of an experiment. The fractional excretion of Na and K can be determined from the concentration of Na and K in plasma and urine.

The conceptual understanding of how to measure renal function can easily be demonstrated in a short lab designed to allow students to actively participate in some aspects of the experiment. This video depicts the pre-lab preparation, the renal function demonstration, and the post-lab evaluation of results. The surgical techniques necessary for making measurements of GFR are demonstrated in an anesthetized rat. In addition, example calculations for GFR, and the fractional excretion of Na and K are shown before and after administration of a diuretic drug.

PROTOCOL:

Prior to any animal procedure, the institutional animal care and use committee (IACUC) must approve the protocol. This protocol was approved by the Michigan State University IACUC.

1. Pre-Lab Preparation of FITC-inulin solution

1.1) Warm 20 mL of saline to 70 °C and slowly stir in 100 mg of FITC-inulin (5 mg/mL FITC-inulin) until all inulin is dissolved.

1.2) Cool solution to room temperature and add 800 mg of bovine serum albumin (40 mg/mL BSA, lyophilized powder, essentially globulin free, low endotoxin, ≥98% purity by agarose gel electrophoresis).

1.3) Filter the inulin-BSA solution with filter paper (grade 1). Place the filtered solution in a 20 mL syringe with a syringe-tip filter (0.2 micron) and cover with foil to protect from light.

2. Anesthesia and surgery

2.1) Place the rat in an induction chamber filled with 5% isoflurane to induce anesthesia. Record body weight (250-350 g) and place the rat on a heated surgical platform designed to maintain 37 °C body temperature throughout the experiment. Gently secure the rat to the platform with laboratory tape over the paws.

2.1.1) Maintain anesthesia with 1-2% isoflurane at airflow rate of 0.8 – 1.0 L/min.

2.2) Insert a tapered catheter into the femoral artery for blood pressure and heart rate monitoring, and blood sampling. Secure the catheter to surrounding tissue with suture. Attach the catheter to a strain gauge pressure transducer. Record blood pressure and heart rate using data acquisition software and display on a computer screen in real-time. This technique is demonstrated in detail on video [6].

2.3) Insert a catheter (PE-50) into the femoral vein for inulin infusion. Secure the catheter to surrounding tissue with suture[6].

2.4) Expose the bladder via a suprapubic incision. Cut a small hole in the tip of the bladder and insert a cannula (PE-190) with a heat flared tip inside the bladder for urine collection. Secure the cannula to the bladder with a purse-string suture.

3. Urine and blood collection

3.1) Place the syringe of FITC-inulin in a syringe pump with flow rate set of 1 mL/h per 100 g of body weight (3 mL/h for a rat weighing 300 g). Attach the syringe to the femoral vein catheter. Start the inulin infusion and allow a 1-2 hour equilibration period. Keep syringe covered with foil to protect from light.

3.2) Determine if urine flow rate is stable and adequate for sample analysis (20 µL/min) by collecting a urine sample in a pre-weighed collection vial for a period of 10 minutes. Determine urine volume gravimetrically with a digital scale. An adequate urine volume for a 10 minute collection period is 0.2 mL. Continue to collect urine samples until two consecutive collections indicate a urine flow rate of 20 µL/min or more.

3.3) Pre-Drug Samples:

3.3.1) Collect a urine sample during a 20 minute period. Collect a blood sample (0.5 mL) from the arterial catheter at the midpoint of the urine collection period. Be careful to completely clear the arterial catheter of saline before collecting a blood sample in a collection vial containing 1 U heparin. Use collection vials with volume markings to facilitate the collection of 0.5 mL of arterial blood.

3.3.2) Flush the arterial catheter with heparin-saline (20 U/mL) to clear the catheter of blood (approx. 0.1 mL). The length of the arterial catheter should be as short as possible to limit the volume of heparin-saline required to flush.

Note: Diluted blood samples produce inaccurate calculations of GFR and fractional excretion of Na and K.

3.3.3) Wait 10 minutes, and repeat the collection of a second Pre-Drug urine and blood sample.

3.4) Following the collection of two Pre-Drug samples, administer a diuretic drug, furosemide (10 mg/kg), via the arterial catheter. Flush the arterial catheter with heparinized saline to clear the catheter of drug. Take care to prevent the injection of air through the arterial catheter. Record the time of the furosemide injection.

3.5) Post-Drug Samples: At each of the 3 time points below, collect a urine sample during a 10 minute collection period, and a blood sample (0.5 mL) at the midpoint of the urine collection period.

3.5.1) For Post-Drug Sample 1 – collect five minutes after furosemide.

3.5.2) For Post-Drug Sample 2 – collect ten minutes after furosemide.

3.5.3) For Post-Drug Sample 3 – collect fifteen minutes after furosemide.

3.6) After all samples have been collected, euthanize the rat in accordance with institutional procedures by thoracotomy and removal of the heart. Remove both kidneys. Decapsulate (remove the surrounding membrane) and blot the kidneys to remove excess blood. Weigh the kidneys.

4. Sample Analysis

4.1) Measure all urine sample volumes gravimetrically with a digital scale, and record weights.

4.2) Centrifuge whole blood samples with a table-top centrifuge (1,800 x g) to separate plasma. Transfer plasma samples to small labeled vials.

4.3) Analyze Na and K concentrations in urine and plasma samples with a sodium/potassium analyzer.

4.4) Measurement of FITC-inulin in plasma and urine

4.4.1) Dilute pre-drug urine (from 1:200 to 1:400), and post-drug urine (1:10) with HEPES buffer (500 mM, pH 7.4).

4.4.2) Add 40 µL of standard or sample and 60 µL of HEPES buffer in a 96 well plate (one sample per well) and allow to mix for 10 minutes while covered with aluminum foil.

4.4.3) Generate a standard curve for FITC-inulin for concentrations of 6.25, 12.5, 25, 50, 100, 200, 400 µg/mL (Figure 1). Determine FITC-inulin fluorescence in samples and standards using a microplate reader with excitation and emission wavelengths of 485 and 538 nm, respectively.

4.4.4) Fit the fluorescent values for the standards to a 4-paramter logistic function regression analysis. The regression function parameters are used to calculate FITC-inulin concentration in plasma and urine samples (Table 1).

5. Post-Lab Analysis of Results: Calculations

5.1) Calculate Urine Flow Rate (UV; mL/min): [volume of urine collected (mL)] ÷ [time of collection (min)]

5.2) Calculate Glomerular Filtration Rate (GFR; mL/min): [Urine inulin concentration (µg/mL) x UV (mL/min)] ÷ [Plasma inulin conc. (µg/mL)]

5.3) Calculate Filtered Sodium Load (µmol/min): Plasma sodium concentration (µmol/mL) x GFR (ml/min)

5.4) Calculate Sodium Excretion Rate ($U_{Na}V$; µmol/min): Urine sodium concentration (µmol/mL) x UV (ml/min)

5.5) Calculate Fractional Excretion of Sodium (FE Na; %): [$U_{Na}V$ (µmol/min)] ÷ [Filtered Sodium Load (µmol/min)] x 100

5.6) Calculate Filtered Potassium Load (µmol/min): Plasma potassium concentration (µmol/mL) x GFR (mL/min)

5.7) Calculate Potassium Excretion Rate (U_KV; µmol/min): Urine potassium concentration (µmol/mL) x UV (mL/min)

5.8) Calculate Fractional Excretion of Potassium (FE K; %): [U_KV (µmol/min)] ÷ [Filtered Potassium Load (µmol/min)] x 100

REPRESENTATIVE RESULTS:

The diuretic used in the lab demonstration was furosemide which very quickly inhibits the reabsorption of Na and K filtered by the kidney resulting in increased Na, K, and water excretion within minutes of drug administration. By its primary mechanism, furosemide should have minimal effects on GFR and the filtered load of Na and K, but will increase urine flow, and fractional excretion of Na and K.

The representative results in Table 3 show that in an anesthetized rat, the average of the pre-drug values for GFR was 3.2 mL/min, Na excretion was 0.58 µmol/min (0.1% of the filtered load),

and K excretion was 4.4 μmol/min (27% of the filtered load). Five minutes after furosemide (post-drug 1), GFR and the filtered load of Na and K were unaffected. However, the fractional excretion of Na increased to 11.5%, and the fractional excretion of K increased to 63% of the respective filtered loads. The measurements of MAP and HR indicate that furosemide had minimal effects on MAP and HR (Table 2).

The indices of renal function assessed in the laboratory demonstration were the GFR, defined as the rate by which plasma is filtered by the kidney; the filtered Na and K, defined as the rate by which Na and K are filtered by the kidney; the Na and K Excretion Rate, defined as the rate by which Na and K are excreted by the kidney; and the fractional Excretion of Na and K, defined as the percentage of filtered Na and K that is excreted by the kidney

Figure 1 Title: Inulin Standard Curve
FITC fluorescence values are shown for standards containing 6.25, 12.5, 25, 50, 100, 200 and 400 μg/mL inulin. A 4-paramter logistic function regression analysis generates the best-fit curve. The regression function parameters from this curve were used to calculate FITC-inulin concentration in plasma and urine samples.

Table 1: Sample results of inulin assay
FITC-Inulin fluorescence values are shown for the reagent blank, 7 standards, and 5 urine samples. Standards and samples were assayed in duplicate and diluted as needed. The average fluorescence for each sample was used to calculate the concentration of inulin. The inulin concentrations in these urine samples are included in the table of measurements (Table 2).

Table 2: Measurements recorded during the renal function lab demonstration
The variables recorded during five time periods (two Pre-drug and three Post–drug) of the renal function lab demonstration are right and left kidney weight, mean arterial pressure (MAP), heart rate (HR), sample time, urine volume, plasma and urine sodium (Na), potassium (K), and inulin concentrations. The urine inulin concentrations were determined from the inulin assay shown in Table 1.

Table 3: Renal function parameters calculated from recorded measurements
Using the formulas shown in Protocol Section 5, the recorded variables (Table 2) are used to calculate urine flow rate, glomerular filtration rate (GFR), GFR/g kidney weight, excretion rate, filtered load, and fractional excretion of sodium (Na) and potassium (K) during the two Pre-drug and three Post–drug periods.

DISCUSSION:
An appropriate marker for GFR measurement must meet four criteria: be freely filtered at the glomerulus, be unbound to plasma proteins, and neither be absorbed nor secreted in the nephron. Inulin is a fructose polymer which satisfies these criteria. As a result, the renal clearance of inulin is considered the gold standard for measuring GFR [7]. The demonstrated technique represents the traditional approach of determining the renal clearance of inulin using timed urine collections during a constant infusion of inulin [8,9]. Traditional inulin measurements have been made using the anthrone method to produce a quantitative colorimetric determination of inulin measured by spectrophotometer [10,11]. However, in an attempt to facilitate the measurement of

inulin in smaller volumes of urine and plasma, inulin has been tagged with radioactive[12-14], and fluorescent labels[15-17]. The lab demonstration presented in this video used FITC labelled inulin for the measurement of renal function because of the lack of risk of human radiation exposure and the ease of measuring FITC fluorescence[15].

This lab demonstration is intended to provide a conceptual understanding of how to measure renal function to students with minimal laboratory skills. Therefore, 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. At this time, the students are presented with a Pre-Lab overview and informed of the procedures that have been conducted on the animals. Two students are assigned to one animal experiment, and instructed on how to collect blood and urine samples before and after the administration of the diuretic drug. The analysis of blood and urine samples is conducted by experienced technicians and results are delivered to the students for calculations of renal function. Results are presented during a Post-Lab discussion which can be scheduled after the demonstration.

There are several critical steps within the protocol to insure valid responses. First, FITC inulin must be completely dissolved and filtered prior to animal administration. Ideally, FITC inulin should be dialyzed in water for 48 hours at room temperature to remove residual unbound FITC. Second, plasma samples must be free of saline. Students are instructed to collect a blood sample only after all of the saline in the arterial catheter has been expelled and only blood is flowing out of the catheter. Blood samples that are diluted with saline will provide inaccurate values for plasma inulin, sodium and potassium. Second, urine flow must be steady and adequate to produce enough sample for analysis. A steady urine flow rate at baseline is critical because it is an indication of a stable experimental preparation. If urine flow is too low, the infusion rate of inulin can be increased prior to sample collections. However, the infusion of inulin must be constant during the course of the experiment, i.e., inulin infusion rate should not be adjusted during the experiment. Finally, the measurement of inulin fluorescence in plasma and urine samples by microplate reader is critical to a successful experiment. Since the specifications of the microplate reader will determine if samples require dilutions, it is recommended that a test run of the inulin assay be conducted prior to the lab demonstration in an effort to optimize the specifications of the microplate reader and ensure that sample fluorescence values are within the mid-range of the standard curve.

While assessing renal function based on the renal clearance of inulin is considered the gold standard, this technique has limitations because the animals must be anesthetized, and instrumented with vascular and bladder catheters. Anesthethetic agents have been shown to affect renal hemodynamics and GFR [18,19]; however isoflurane and inactin are typically used in renal function experiments due to their minimal effects on the kidney[19,20]. The inulin clearance technique also requires a constant infusion of inulin and multiple blood and plasma samples which can be prohibitive in smaller animals such as mice. Modifications of this technique have been developed to allow the measurement of plasma clearance from a single injection of inulin in conscious animals [21]. These modifications also require smaller volumes of blood samples for analysis, and provide an alternate method for assessing renal function in mice.

The measurement of renal function is applicable to studies of physiology, pathology, toxicology, pharmacology and disease states. Students who participate in the Renal Function demonstration will learn the gold standard technique of renal clearance of inulin to assess renal function. By mastering this technique, students will understand the principles of renal function and allow them to apply the technique to their own research and determine if modifications to the technique are appropriate for their studies.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure
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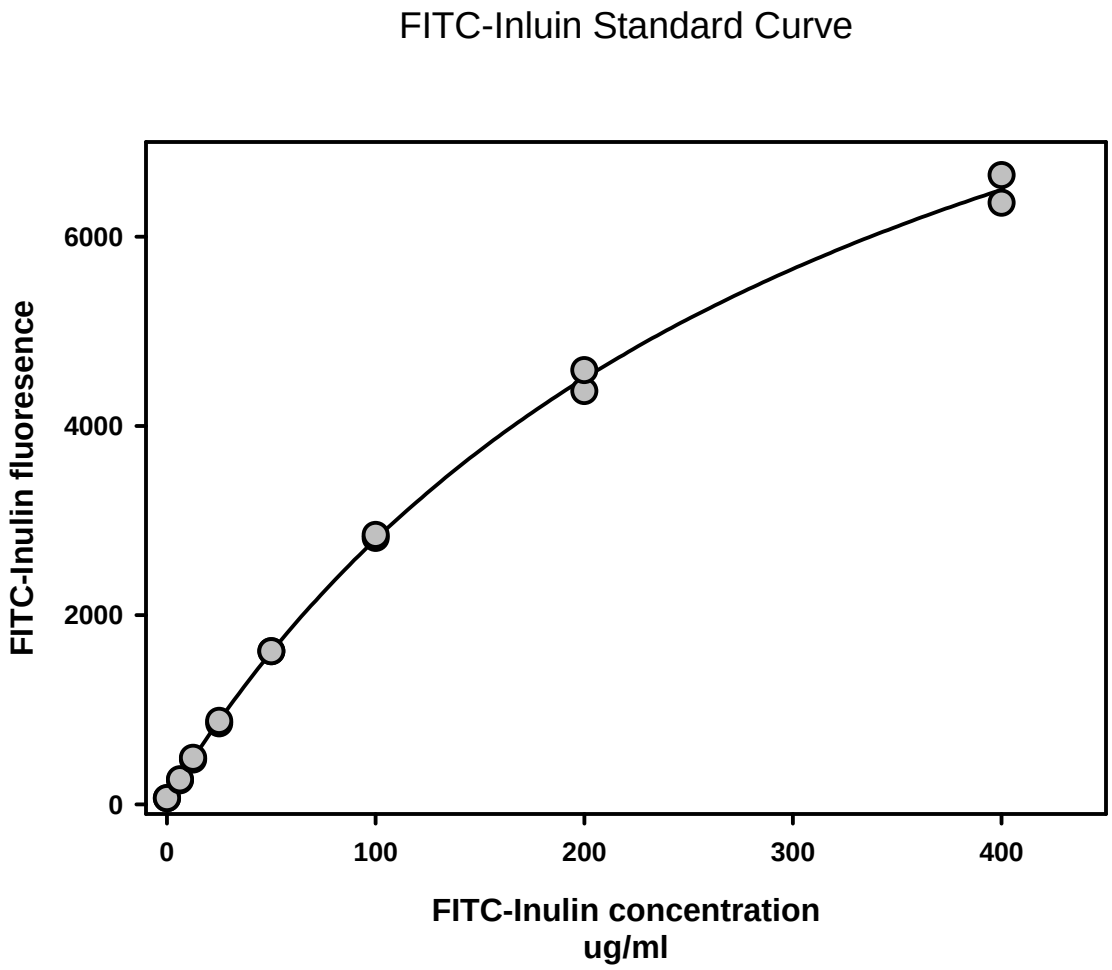

Table 1

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TABLE 1: Inulin Standard Curve

	FITC-Inulin fluoresence			Concentration		Result
Standard	replicate 1	replicate 2	Mean	µg/ml	Dilution	µg/ml
Blank	63.9	64.8	64.4	0.4	1	0.4
6.25	253.2	264.1	258.7	5.9	1	5.9
12.5	474.0	491.3	482.7	12.5	1	12.5
25	854.8	881.3	868.1	24.4	1	24.4
50	1617.1	1618.0	1617.6	50.3	1	50.3
100	2813.1	2846.1	2829.6	101.3	1	101.3
200	4367.3	4588.7	4478.0	198.2	1	198.2
400	6258.0	6650.0	6454.0	401.6	1	401.6
Urine Sample						
Pre-drug 1	2443.9	2062.3	2253.1	88.5	200	17700
Pre-drug 2	2266.5	1707.0	1986.8	76.3	200	15250
Post-drug 1	1208.9	1391.2	1300.1	44.7	10	447
Post-drug 2	2753.4	2120.5	2437.0	97.0	10	970
Post-drug 3	2888.3	3178.0	3033.2	124.4	10	1244

Table 2
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TABLE 2: Measurements

Experiment Time Point	MAP (mm Hg)	HR (beats /min)	Duration of Sample Collection (min)	Urine Vol. (ml)	Plasma Na (µmol/ml)	Urine Na (µmol/ml)
Pre-drug 1	98	376	20	0.20	141	49
Pre-drug 2	100	376	20	0.30	142	45
Post-drug 1	107	376	10	5.80	143	110
Post-drug 2	109	387	10	3.10	142	117
Post-drug 3	108	372	10	2.00	143	117

Plasma K (µmol/ml)	Urine K (µmol/ml)	Plasma Inulin (µg/ml)	Urine Inulin (µg/ml)	right and left kidney weight (g)
5.1	375.0	63.4	17700	2.1
5.0	341.0	62.9	15250	
4.6	19.5	66.9	447	
4.0	26.4	62.6	970	
3.7	33.5	64.0	1244	

Table 3
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TABLE 3: Calculations

	Urine Flow (ml/min)	GFR (ml/min)	GFR/g Kidney wt (ml/min/g)	Na Excretion (µmol/min)	Filtered Na (µmol/min)	Fractional Excretion of Na (%)	K Excretion (µmol/min)
Pre-drug 1	0.01	2.8	1.3	0.49	393.6	0.12	3.75
Pre-drug 2	0.02	3.6	1.7	0.68	518.1	0.13	5.12
Post-drug 1	0.58	3.9	1.8	63.80	554.2	11.51	11.31
Post-drug 2	0.31	4.8	2.3	36.27	682.1	5.32	8.18
Post-drug 3	0.20	3.9	1.9	23.40	555.9	4.21	6.70

Filtered K (μmol/min)	Fractional Excretion of K (%)
14.24	26.34
18.24	28.04
17.83	63.44
19.21	42.59
14.38	46.58

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: JoVE_Materials for Renal Function Lab-alpha order.xls

Name of Material/ Equipment	Company	Catalog Number
5-0 Braided Silk Surgical Suture	Surgical Specialties Corp	SP1033
Assay Plate, 96-Well	Costar	3922
Bovine Serum Albumin	Sigma Chemical Co	A2934-25G
Centrifuge	Beckman Coulter	MicroFuge 18, 357160
Conical Sample Tubes	Dot Scientific Inc.	#711-FTG
Cotton Tipped Applicators	Solon Manufacturing Co	56200
Data Acquisition Software	ADInstruments	LabChart Pro 7.0
Digital Scale	Denver Instrument	APX-4001
FITC-Inulin	Sigma Chemical Co	F3272-1G
Gauze Sponges	Covidien	2146
Heated Surgical Bed	EZ-Anesthesia	EZ-212
Heparin	Sagnet	NDC 25021-402-10
HEPES	Sigma Chemical Co	H3375
Isoflurane	Abbott Animal Health	IsoFlo, 5260-04-05
Isoflurane Vaporizer	EZ-Anesthesia	EZ-190F
Micro Dissecting Forceps	Biomedical Research Instruments Inc.	70-1020
Microplate Reader - Fluoroskan	ThermoScientific	Ascent FL, 5210460
NOVA 5+ Sodium/Potassium Analyzer	NOVA BioMedical	14156
Olsen-Hegar Needle Holders with Scissors	Fine Science Tools	12002-12
PE-190 (for bladder catheter)	BD Medical	427435
Pressure Transducer	ADInstruments	MLT1199
Pyrex Culture Tubes	Corning Inc.	99445-12
Rat Femoral Tapered Artery Catheter	Strategic Applications Inc.	RFA-01
Salix Furosemide 5%	Intervet	#34-478
Strabismus Scissors	Fine Science Tools	14075-11
Student Surgical Scissors	Fine Science Tools	91402-12
Surgical Gloves	Kimberly-Clark	Sterling Nitrile Gloves
Syringe pump	Razel Scientific	R99-E
Tissue Forceps	Fine Science Tools	91121-12
Tissue Scissors	George Tiemann Co	105-420

Comments/Description

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Author(s): Carmen Hingosa-Laborde, Brian Jespersen, Robert E. Shade

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Response to Editor's Comments
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Dear JoVE editors,

Thank you for editorially reviewing our manuscript JoVE52425 'Physiology Lab Demonstration: Renal Function in a Rat'.

We have addressed your comments below. We have used the "track-changes" function in Microsoft Word to identify all of our manuscript edits. We have also uploaded this document labeled, 'Response to Editor's Comments". Thank you for your comments. We are confident that your suggestions for revisions have significantly improved the quality of our submitted manuscript.

Editorial comments:

1.Please state the goal of your protocol in the Short abstract.

Response: The Short Abstract has been revised to "Assessing renal function is critical in evaluating the renal effects of drugs and disease. The purpose of this protocol is to demonstrate the principles and techniques for measuring and calculating glomerular filtration rate, urine flow rate, and excretion of sodium and potassium a presented as a hands-on teaching laboratory.

2.Please specify the pore size of the filter in step 1.3 and please remove the word "Millipore".

Response: The pore size has been included and the word "Millipore" has been removed from the revised manuscript.

3.Please specify the dose of isoflurane in step 2.1.

4.Is the animal secured before cannula implantation? If so please specify.

Response: In response to comments #3 and #4, Step 2.1 has been revised to, "Induce anesthesia in the rat with 5% isoflurane. Record body weight and place the rat on a heated surgical platform to maintain 37 °C body temperature. Gently secure the rat to the platform with laboratory tape over the paws. Maintain anesthesia with 1.8% isoflurane."

5.Please write step 2.2 in imperative tense, "Record the blood pressure and heart rate…".

Response: Corrected version: "Record blood pressure and heart rate using data acquisition software and display on a computer screen in real-time [5].

6.Please write the "Post-Drug sample 1, 2, 3, in a sentence format.

Response: Corrected version:

Collect Post-Drug Sample 1 five minutes after furosemide.

Collect Post-Drug Sample 2 ten minutes after furosemide.

Collect Post-Drug 3 fifteen minutes after furosemide.

7.Please write step 4.1 in imperative tense. “Measure…”, “Centrifuge…” Additionally please specify the centrifugation speed in “ x G” and the time.

Response: Corrected version: “Measure urine volume gravimetrically with a digital scale. Centrifuge whole blood samples with a table-top centrifuge (18,000 x G) to separate plasma. Transfer plasma samples to small labeled vials.

8.Please mention step 4.2 in imperative tense. “Analyze Na and K concentrations..”

Response: Corrected version: “Analyze Na and K concentrations in urine and plasma samples with sodium/potassium analyzer.”

9.Please split step 4.3 in to smaller steps such that each step contains only two to three action items per step.

Response: This step has been divided into 4 smaller steps.

4.4) Measurement of FITC-inulin in plasma and urine

4.4.1) Dilute pre-drug urine (from 1:200 to 1:400), and post-drug urine (1:10) with HEPES buffer (500 mM, pH 7.4).

4.4.2) Add 40 ul of standard or sample and 60 ul of HEPES buffer in a 96 well plate (one sample per well) and allow to mix for 10 minutes while covered with aluminum foil.

4.4.3) Generate a standard curve for FITC-inulin for concentrations of 6.25, 12.5, 25, 50, 100, 200, 400 µg/ml (Figure 1). Determine FITC-inulin fluorescence in samples and standards using a microplate reader with excitation and emission wavelengths of 485 and 538 nm, respectively.

4.4.4) Fit the fluorescent values for the standards to a 4-paramter logistic function regression analysis and the regression function parameters are used to calculate FITC-inulin in plasma and urine samples (Table 1).

10.Please write section 5 in correct number format. And please define what each of the terms in the formula stand for.

Response: Section 5 has been revised to show the correct number format. All term is the formulas are defined.

5. Post-Lab Analysis of Results: Calculations

5.1) Urine Flow Rate (UV; ml/min) = $\frac{\text{volume of urine collected (ml)}}{\text{time of collection (min)}}$

5.2) Glomerular Filtration Rate (GFR; ml/min) $= \frac{\text{Urine inulin conc. (µg/ml) x UV (ml/min)}}{\text{Plasma inulin conc. (µg/ml)}}$

5.3) Filtered Sodium Load (uM/min) = Plasma sodium concentration (µM/ml) x GFR (ml/min)

5.4) Sodium Excretion Rate ($U_{Na}V$; uM/min) = Urine sodium concentration (µM/ml) x UV (ml/min)

5.5) Fractional Excretion of Sodium (FE Na; %) = $\frac{U_{Na}V\ (\mu M/min)}{\text{Filtered Sodum Load } (\mu M/min)}$ X 100

5.6) Filtered Potassium Load (uM/min) = Plasma potassium concentration (µM/ml) x GFR (ml/min)

5.7) Potassium Excretion Rate (U_KV; uM/min) = Urine potassium concentration (µM/ml) x UV (ml/min)

5.8) Fractional Excretion of Potassium (FE K; %) = $\frac{U_KV\ (\mu M/min)}{\text{Filtered Potassium Load } (\mu M/min)}$ X 100

11.In step 4.3 since you are measuring the FITC fluorescence in sample, it would be ideal to have a data showing the standard curve and the bar graph or table with the resulting concentration.

Response: A figure of the standard curve (Figure 1) and a table of resulting concentrations (Table 1) have been included in the revised manuscript. The original tables have been re-numbered to Table 2 and Table 3.

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Response: The manuscript has been revised to remove all commercial sounding language. All commercial products are referenced in the table of materials.

13.After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is no page limit for the protocol text, but there is a 3 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight (in yellow) 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Response: The Protocol Section is less than 3 pages.

14.Please provide a figure legend as per JoVE format for the figure, including title, description.

Response: All figures and tables have a title and legend in the revised manuscript.

15.Please make sure that the "Discussion" section covers the following pints running between 3 – 6 paragraphs.

a.Critical steps within the protocol.

b.Modifications and troubleshooting.

c.Limitations of the technique.

d.Significance of the technique with respect to existing/alternative methods.

e.Future applications or directions after mastering this technique.

Response: The revised Discussion section now covers the 5 points outlined above.

16.Issue number and DOI's are missing. Please make sure that your references comply with JoVE instructions for authors. In-text formatting: corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text of the manuscript. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI, (YEAR).]

Response: The authors have included the issue numbers and the DOI's for the references which had this information available. Some references did not have issue numbers and/or DOI numbers.

Rebuttal Comments - Response

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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.

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.

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.

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.

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.