Journal of Visualized Experiments

Transdermal measurement of glomerular filtration rate in mice

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58520R2
Full Title:	Transdermal measurement of glomerular filtration rate in mice
Keywords:	Glomerular filtration rate, FITC-Sinistrin, transdermal, mice, rodents, Kidney Function
Corresponding Author:	Daniel Schock-Kusch MediBeacon GmbH Mannheim, Baden Württemberg GERMANY
Corresponding Author's Institution:	MediBeacon GmbH
Corresponding Author E-Mail:	dschock-kusch@medibeacon.com
Order of Authors:	Lauren Scarfe
	Daniel Schock-Kusch
	Jochen Friedemann
	Yury Shulhevich
	Lorenzo Ressel
	Patricia Murray
	Bettina Wilm
	Mark de Caestecker
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Division of Nephrology S-3223, Medical Center North 1161 21st Street S Nashville, TN 37232-2372

TITLE

Transdermal Measurement of Glomerular Filtration Rate in Mice

AUTHORS AND AFFILIATIONS

Lauren Scarfe[1, 2], Daniel Schock-Kusch[3], Lorenzo Ressel[4], Jochen Friedemann[3], Yury Shulhevich[3], Patricia Murray[2], Bettina Wilm[2], Mark de Caestecker[1]

[1]Division of Nephrology, Department of Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA
[2]Department of Cellular and Molecular Physiology, University of Liverpool, Liverpool, UK
[3]MediBeacon GmbH, Mannheim
[4]Department of Veterinary Pathology and Public Health, Institute of Veterinary Science, University of Liverpool, Liverpool, UK

Corresponding Authors:

Daniel Schock-Kusch Ph.D. (dschock-kusch@medibeacon.com)
Tel.: +49 (0)621-150283-15

Bettina Wilm Ph.D. (b.wilm@liverpool.ac.uk)

Mark de Caestecker M.B., B.S., Ph.D., F.A.S.N. (Mark.de.Caestecker@vanderbilt.edu)

Email Addresses of Co-Authors:
Lauren Scarfe (lauren.scarfe@vumc.org)
Lorenzo Ressel (L.Ressel@liverpool.ac.uk)
Jochen Friedemann (jfriedemann@medibeacon.com)
Yury Shulhevich (yshulhevich@medibeacon.com)
Patricia Murray (P.A.Murray@liverpool.ac.uk)

KEYWORDS
Glomerular filtration rate, FITC-sinistrin, transdermal, mice, rodents, kidney function

SUMMARY
Here we describe a protocol to measure glomerular filtration rate (GFR) in conscious, freely moving mice using a transdermal GFR monitor.

ABSTRACT
Transdermal analysis of glomerular filtration rate (GFR) is an established technique that is used to assess renal function in mouse and rat models of acute kidney injury and chronic kidney disease. The measurement system consists of a miniaturized fluorescence detector that is directly attached to the skin on the back of conscious, freely moving animals, and measures the excretion kinetics of the exogenous GFR tracer, fluorescein-isothiocyanate (FITC) conjugated

sinistrin (an inulin analog). This system has been described in detail in rats. However, because of their smaller size, measurement of transcutaneous GFR in mice presents additional technical challenges. In this paper we therefore provide the first detailed practical guide to the use of transdermal GFR monitors in mice based on the combined experience of three different investigators who have been performing this assay in mice over a number of years.

INTRODUCTION

The use of transcutaneous GFR monitors in mice was first reported by Schreiber and colleagues in 2012 and was validated by comparing GFR measurements obtained using this technique, with results obtained by direct measurement of FITC-sinistrin bolus clearance from serial blood samples[1]. To date, there have been 35 peer-reviewed publications in which transcutaneous GFR monitors have been used in rats and mice (a regularly updated list of journal articles and conference abstracts in which the preclinical GFR monitor was used can be found at the MediBeacon website[2]). Transdermal GFR measurements in rats and mice has been described in a number of publications[1,3-5], and a video tutorial demonstrating its use in rats has been published[6]. However, measurement in mice presents additional technical challenges. Here, we provide the first detailed practical guide to the use of transdermal GFR monitors in mice.

There are a variety of reasons why investigators are starting to favor the use of transdermal GFR monitors to assess renal function in rodent models. Transdermal measurement of FITC-sinistrin clearance has been shown to provide a more sensitive and accurate measure of renal function compared to the traditional parameters of renal function such as serum creatinine and blood urea nitrogen (BUN)[7,8]. By implementing an improved evaluation algorithm, Friedemann and colleagues demonstrated that the system reaches precision comparable to the gold standard, the constant infusion technique for GFR measurement[3]. Recent studies have also shown that sequential analysis using transcutaneous GFR monitors can be used to study early changes in renal function as well as functional recovery after induction of acute kidney injury (AKI) without interfering with the animals' blood volume or hemodynamics, since the assay does not require sequential blood sampling[9,10]. The ability to measure GFR with high precision and sensitivity repeatedly in the same animal makes this technique attractive for a variety of different research disciplines. Transdermal GFR monitors have been used by pharmaceutical companies to assess the toxicity of novel compounds, as well as in universities for basic and translational research.

PROTOCOL

All animal experiments were performed in accordance with local guidelines in the UK and USA. Experiments conducted at the University of Liverpool were performed under a license granted under the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of Liverpool ethics committee. All animal experiments conducted at Vanderbilt University Medical Center were approved by the Vanderbilt Institutional Animal Care and Use Committee.

1. Preparing the FITC-sinistrin

1.1. Prepare 40 mg/mL FITC-sinistrin in phosphate buffered saline (PBS).

Note: Aliquots can be stored at -20 °C for several months with no noticeable decrease in quality; however multiple freeze-thaw cycles should be avoided. FITC-sinistrin is light sensitive - keep the tube protected from light.

1.2. Calculate the volume of FITC-sinistrin required for each mouse:

1.2.1. Weigh each mouse on each day of measurement.

1.2.2. The recommended dose is 0.15 mg FITC-sinistrin per gram body weight.

2. Mouse Preparation

2.1. Prepare separate cages for the mice while undergoing GFR measurements. Provide absorbent paper towels and a few pellets of food.

3. Removing Hair from the Mouse (1-2 days before GFR Measurement)

3.1. Anesthetize the mouse with 3% isoflurane, and once the mouse is asleep, maintain anesthesia with 1.5-2% isoflurane, depending on the breathing rate of the mouse. Place the mouse prone on a heat pad.

3.2. Use an electric shaver, going against the direction of the fur, to remove most of the fur from one side of the mouse's back. Shave from the top of the hind legs up to the neck, and across the ribs.

3.3. Apply a thin layer of depilation cream to the shaved area using a cotton bud (**Figure 1A**). Move the cotton bud against the direction of the fur to ensure that the cream is applied as close to the skin as possible.

3.4. Remove the cream after 1-3 min by washing it off with cotton swabs and warm water. Do not perform the measurement if the skin appears very red and irritated after measurement, and do not repeat depilation within 72 h to avoid damaging the skin.

4. Preparing the Transdermal GFR Monitor

4.1. Use one of the two sizes of patches that are available. The first is 2.5 x 3 cm in size and can be used for measurements in mice directly. The other patches are 6 x 3 cm in size and are meant to be used in rats or larger animals but can be cut to a smaller size for use in mice.

4.2. Peel the backing off one side of the patch and stick the GFR device on the adhesive side, positioning the LEDs exactly above the clear window.

4.3. Cut the excess adhesive patch to fit the size of the battery and stick one side of the patch to the battery.

5. Attaching the Transdermal GFR Monitor

5.1. Anaesthetize the mouse with isoflurane as described in step 3.1 and place the mouse prone on a heat pad. Anesthetize mice only for placement of the transdermal GFR monitor and injection of FITC-sinistrin; allow to recover from anesthesia for the measurement of FITC decay.

5.2. Clean the pre-shaved skin with 70% ethanol. Place approximately 12 cm of tape under the mouse (**Figure 1B**; the width of the tape should be reduced to 1.5-2 cm so that it is not too wide for the mouse).

5.3. Position the tape so that only approximately 2 cm is on the mouse's right side, and the rest is on the left. Fold over one edge of the right side of tape for easy placement and removal after the measurement. The left-right instructions for steps 5.3 and 5.6 are for device placement on the right side of the animal and can be swapped for device placement on the left side of the animal if required.

5.4. Connect the battery to the device, remove the backing from the battery and securely place it on top of the device. The device is ready to use and data acquisition starts when the blue light emitting diodes (LEDs) start blinking.

5.5. Remove the backing from the device and place on to the shaved skin. Position the device such that the window exposing the LEDs is over the ribs – do not have it too close to the spine or limbs (**Figure 1C**).

5.6. Secure the device with the white tape. Secure the right side first (**Figure 1D**), wrapping it tightly around all edges of the device, then wrap the left side around the mouse and device (**Figure 1E**). Ideally, the left side of the tape only covers the device, and the right side ends under the mouse's abdomen.

5.7. Attach the tape by pressing it alongside the circumference of the mouse's body. The tape needs to be attached firmly, but not tightly. If it is too loose then the device will move around too much and cause movement artefacts. However, it should not be so tight that it restricts breathing or movement or puts too much pressure on the skin.

5.8. Leave the device untouched for 3 minutes before the FITC-sinistrin injection to allow a steady background reading to be taken. In this time, warm the tail with a heat pad or glove filled with warm water to prepare for tail vein injection (if using this route).

6. FITC-Sinistrin Injection

6.1. Prepare an insulin syringe with the calculated amount of FITC-sinistrin required for injection (this can be rounded to the nearest 10 µL).

6.2. Administer FITC-sinistrin by tail vein or retro-orbital injection. FITC-sinistrin should be administered in one smooth but rapid bolus to avoid multiple peaks on the clearance curve. It is better to administer only a partial dose than to have multiple attempts at administering the FITC-sinistrin.

7. Measuring the GFR

7.1. Place the mouse in a cage on its own to recover from isoflurane anesthesia and for the duration of the measurement period.

7.2. Observe the mouse in the cage for 1.5 h and then remove the device. Removing the device from the conscious mouse is fast, efficient, and generally well-tolerated by the mouse, but new users may prefer to anaesthetize the mouse for this step.

7.2.1. As one option, anaesthetize the mouse with isoflurane.

7.2.2. As the other option, place the mouse on the wire rack on top of the cage, allowing the mouse to grasp the metal bars whilst the device is removed.

7.3. Pull off the white plaster tape from underneath the belly in one quick, smooth movement, and remove the device and black plaster from the skin. Be careful that the battery does not disconnect from the device yet.

7.4. Return the mouse to its home cage.

8. Reading and evaluating the data

8.1. Carefully disconnect the battery from the device

8.2. Connect the device to the USB cable and then connect the cable to the computer

8.3. Open the reading software (Sensor_ctrl_app.exe)

8.4. In order, click “connect”, “read”, “re-name”, and “save”, then close the program

8.5. Process and evaluate data in the analysis software as described in the respective manual

REPRESENTATIVE RESULTS

In this section we present representative results of the use of the transdermal GFR monitor. The transdermal monitor has been used in a variety of mouse strains and models of AKI and CKD[2].

Figure 2 shows representative FITC-sinistrin clearance curves in male BALB/c mice before and after ischemia reperfusion injury (IRI) with simultaneous contralateral nephrectomy. FITC-sinistrin is rapidly cleared from the circulation in healthy mice (**Figure 2A**), but clearance is dramatically delayed in mice with AKI (**Figure 2B,C**). In mice with very severe AKI, there may not be any change in FITC-sinistrin fluorescence during the 90-minute measurement period, indicating a complete absence of glomerular filtration (**Figure 2C**).

Transdermal GFR measurement is minimally invasive and can be used to monitor changes in kidney function in the same mice over multiple time points. **Figure 3** depicts changes in GFR determined by sequential transdermal FITC-sinistrin clearance measurements at baseline, and 1, 2 and 4 days after inducing IRI (unilateral ischemia with simultaneous contralateral nephrectomy). Data shown includes FITC-sinistrin clearance half-life (**Figure 3A**), and GFR (**Figure 3B**) calculated from the measured FITC-sinistrin clearance half-life, as described by Schreiber *et al*[1].

In **Figure 4**, chronic kidney disease (CKD) was induced in male BALB/c mice by performing prolonged unilateral IRI followed by delayed contralateral nephrectomy, as described[11]. GFR was assessed by transdermal FITC-sinistrin clearance on day 26 after the initial IRI. The increase in FITC-sinistrin half-life (**Figure 4A**), and therefore the decrease in GFR (**Figure 4B**), indicates impaired renal function in these mice. These data demonstrate that transcutaneous GFR measurement can be used to measure changes in renal function in mice with CKD.

Figure 5A shows that FITC-sinistrin half-life correlates closely with semi-quantitative histological assessment of tubular injury over the full range of GFR measurements in uninjured mice and in mice with different severities of IRI-induced AKI. In contrast, serum creatinine and blood urea nitrogen (BUN) showed a positive but weaker correlation with FITC-sinistrin clearance (**Figure 5B,C**), indicating that transcutaneous GFR measurements provide a more reliable measure of renal injury (tubular injury scores) following IRI-induced AKI than either serum creatinine or BUN.

FIGURE AND TABLE LEGENDS

Figure 1: Attaching the transdermal GFR monitor. Photographs of hair removal (**A**), placement of the tape under the mouse (**B**), placement of the device on the mouse's skin (**C**), and securing the device by wrapping the tape around the mouse and device (**D-E**)

Figure 2: Example FITC-sinistrin clearance curves in male BALB/c mice before and after ischemia reperfusion injury (IRI) with simultaneous contralateral nephrectomy. Clearance curves at baseline (**A**), and one day after IRI surgery (**B**) in the same mouse, indicating impaired renal function in this mouse. (**C**) Clearance curve from a more severely injured mouse one day after IRI surgery. There was no clearance of FITC-sinistrin during the measurement period, indicating renal failure. Black data points represent raw data, blue lines represent the 3-compartment fit, and green lines represent 95% confidence intervals.

Figure 3: Male BALB/c mice, age 8-10 weeks underwent unilateral ischemia with simultaneous contralateral nephrectomy (n=5). GFR was assessed at baseline and on days 1, 2, and 4 after

surgery, and compared with sham-operated control mice (n=5). FITC-sinistrin half-life in (**A**) was used, along with the body weight of the mice, to calculate GFR in (**B**). Data points represent individual animals, and error bars represent mean and standard error.

Figure 4: Male BALB/c mice age 8-10 weeks underwent unilateral ischemia with delayed contralateral nephrectomy at day 8 (n=5). GFR was assessed by on day 26 and was compared to age-matched healthy control mice (n=5). FITC-sinistrin half-life in (**A**) was used, along with the body weight of the mice, to calculate GFR in (**B**). Data points represent individual animals, and error bars represent mean and standard deviation. Tubular injury was scored 0-50 based on the degree of necrosis and cast formation by a blinded observer (L.R.) on Periodic acid-Schiff-stained kidney sections. This method was adapted from Wang and colleagues[12].

Figure 5: Correlation of three measures of kidney function/damage (histological evaluation of tubular injury (n=39), serum creatinine (n=30) and blood urea nitrogen (BUN) (n=30)) with FITC-sinistrin clearance (half-life). Male BALB/c mice underwent varying periods of unilateral renal pedicle clamping (25-45 min) or sham surgery, with simultaneous contralateral nephrectomy to induce different severity of AKI, and renal function parameters and histopathology were assessed at day 4 after IRI. The tubular injury score showed a strong positive correlation with FITC-sinistrin clearance (**A**; R^2 = 0.88), whereas serum creatinine (**B**), and BUN (**C**) both showed positive but weaker correlation with FITC-sinistrin clearance (R^2 = 0.64 and 0.52, respectively).

DISCUSSION

This manuscript and the accompanying training video provide practical guidelines for the use of transdermal GFR monitors in mice. The most critical steps in the procedure are the correct attachment of the device on the animal's back, and securely wrapping the tape around the abdomen. The best position is either slightly left or right of the midline, over the ribcage. The patch and device need to be firmly attached to the skin, but they should not be so tight that they restrict breathing, movement, or affect skin blood circulation under the device, as this would lead to faulty/inaccurate measurements. In addition, since monitoring occurs in conscious mice after they have recovered from anesthesia, correct placement of the device on the part of the body with lowest interference from movement results in transdermal measurements with little movement artefacts. For this reason, it is important that the device is not placed too close to the upper limbs so that the mice can move their shoulders freely.

Because up to 50% of serum creatinine is excreted by tubular section in mice[13], and because there is increased reabsorption of urea from renal tubules when mice are dehydrated[14], serum creatinine and BUN are poor markers of renal function. However, because of their convenience, these assays continue to be used as the main measure of renal function in preclinical studies of AKI and CKD in mice. However, consistent with major contribution of tubular secretion to creatinine excretion in mice with normal or near normal renal function[13], serum creatinine showed little correlation with FITC-sinistrin clearance at high clearance rates (low FITC-sinistrin half-life), indicating that creatinine is an insensitive measure of renal function in mice with mild kidney injury. In contrast, while BUN correlates well with FITC-sinistrin clearance in mice with mild renal impairment, there is poor correlation between BUN and FITC-sinistrin clearance in

mice with more severe kidney injury (high FITC-sinistrin half-life). This is likely caused by effects of urea reabsorption associated with dehydration in sick animals with severe kidney injury.

A major advantage of the transdermal GFR measurement, compared to all other bolus clearance or constant infusion techniques for GFR measurement, is that it does not require carefully timed blood or urine collections. These can be particularly challenging in mice as they have low total blood volumes and urinary output as compared with rats. Moreover, mice need to be handled only for attachment of the device and injection, but not for multiple venipunctures, as required for classical bolus clearance experiments[15]. The main limitation of transdermal GFR measurement, as compared with bolus clearance methods to measure GFR in mice is that the excretion kinetics are only measured as change in relative fluorescence intensity over time, and not as absolute tracer concentrations. Because of this, it is only possible to measure the rate constant of the single exponential decay of the excretion kinetic, which is a very close estimate of GFR normalized on extracellular volume[16]. To express GFR in mL/min, the extracellular volume of the animal has to be estimated using a conversion factor that was established in prior studies in which simultaneous measurements of plasma concentrations of FITC-sinistrin were performed[1]. However, this conversion factor may not correctly estimate extracellular fluid volumes equally well in all mice, since fluid volume may be affected by a variety of extraneous factors including age, sex, hydration status (which may be affected by surgical interventions as well as kidney injury), and weight[17]. However, unlike the bolus dosing method to assess GFR in mice, transcutaneous GFR measurement is subject to less operator-dependent variability as it is not affected by dosing errors or by errors in timing of blood collections.

Another limitation of the transcutaneous GFR measurement technique is that baseline signal shifts may occur during the course of the measurement due to bleaching of skin fluorophores and the anesthesia required for device attachment and tracer injection. This limitation was addressed by Friedemann and colleagues by implementing a correction algorithm[3]. The implementation of this algorithm led to an improvement in precision of the transdermal technique comparable to a constant infusion technique of GFR assessment.

A frequently asked question is whether skin pigmentation in different mouse strains affects the transdermal FITC-sinistrin clearance. Skin pigmentation reduces the FITC-sinistrin signal intensity since dark pigments absorbs the blue excitation and the green emission signals from FITC-sinistrin measurements. However, the excretion rate of FITC-sinistrin is independent of the overall signal intensity. Furthermore, while the measured signal is lower, the background signal is also lower in pigmented mice. Because the background signal is a mixture of autofluorescence of skin fluorophores and reflection of the excitation light, we have found that the background-to-maximum signal ratio is comparable, or even improved, in pigmented animals. In addition, movement artifacts, which are caused by exposure of the surrounding skin to reflected light, are reduced in pigmented mice since the reflected light is also absorbed by pigmented skin.

In conclusion, the technique we have presented allows precise measurement of GFR in conscious, freely moving mice of all skin types. As the technique is independent of blood sampling, it can be used repeatedly on the same animal for longitudinal observations in CKD models, as well as for

the measurement of rapid changes of GFR that occur after induction of AKI.

ACKNOWLEDGMENTS

This work was supported by the Vanderbilt Center for Kidney Disease (VCKD), and was in part funded by the following grants: DOD PR161028 and R01DK112688 (Mark de Caestecker)

We acknowledge support to LS, PM and BW by the MRC, EPSRC and BBSRC-funded UK Regenerative Medicine Platform "Safety and Efficacy, focussing on Imaging Technologies Hub" (MR/K026739/1).

DISCLOSURES

D S-K, JF and YS are employees at MediBeacon GmbH the manufacturer and distributor of the transdermal GFR monitor.

D S-K and JF are inventors on patents and patent applications for the presented technology.

REFERENCES

1 Schreiber, A. *et al.* Transcutaneous measurement of renal function in conscious mice. *American Journal of Physiology - Renal Physiology.* **303** (5), F783-788, (2012).

2 MediBeacon. *Related Publications*, <http://www.medibeacon.com/preclinical/publications/> (2018).

3 Friedemann, J. *et al.* Improved kinetic model for the transcutaneous measurement of glomerular filtration rate in experimental animals. *Kidney International.* **90** (6), 1377-1385, (2016).

4 Schock-Kusch, D. *et al.* Reliability of transcutaneous measurement of renal function in various strains of conscious mice. *PLoS One.* **8** (8), e71519, (2013).

5 Schock-Kusch, D. *et al.* Transcutaneous assessment of renal function in conscious rats with a device for measuring FITC-sinistrin disappearance curves. *Kidney International.* **79** (11), 1254-1258, (2011).

6 Herrera Pérez, Z., Weinfurter, S. & Gretz, N. Transcutaneous Assessment of Renal Function in Conscious Rodents. *Journal of Visualized Experiments.* 10.3791/53767 (109), 53767, (2016).

7 Cowley, A. W., Jr. *et al.* Progression of glomerular filtration rate reduction determined in conscious Dahl salt-sensitive hypertensive rats. *Hypertension.* **62** (1), 85-90, (2013).

8 Scarfe, L. *et al.* Measures of kidney function by minimally invasive techniques correlate with histological glomerular damage in SCID mice with adriamycin-induced nephropathy. *Scientific Reports.* **5** 13601, (2015).

9 Lazzeri, E. *et al.* Endocycle-related tubular cell hypertrophy and progenitor proliferation recover renal function after acute kidney injury. *Nature Communications.* **9** (1), 1344, (2018).

10 Street, J. M. *et al.* The role of adenosine 1a receptor signaling on GFR early after the induction of sepsis. *American Journal of Physiology - Renal Physiology.* **314** (5), F788-f797, (2018).

11 Skrypnyk, N. I., Harris, R. C. & de Caestecker, M. P. Ischemia-reperfusion model of acute kidney injury and post injury fibrosis in mice. *Journal of Visualized Experiments.*

10.3791/50495 (78), (2013).
12 Wang, W. *et al.* Endotoxemic acute renal failure is attenuated in caspase-1-deficient mice. *American Journal of Physiology - Renal Physiology.* **288** (5), F997-1004, (2005).
13 Eisner, C. *et al.* Major contribution of tubular secretion to creatinine clearance in mice. *Kidney International.* **77** (6), 519-526, (2010).
14 Bankir, L. & Yang, B. New insights into urea and glucose handling by the kidney, and the urine concentrating mechanism. *Kidney International.* **81** (12), 1179-1198, (2012).
15 Qi, Z. *et al.* Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *American Journal of Physiology - Renal Physiology.* **286** (3), F590-596, (2004).
16 Peters, A. M. The kinetic basis of glomerular filtration rate measurement and new concepts of indexation to body size. *European Journal of Nuclear Medicine and Molecular Imaging.* **31** (1), 137-149, (2004).
17 Chapman, M. E., Hu, L., Plato, C. F. & Kohan, D. E. Bioimpedance spectroscopy for the estimation of body fluid volumes in mice. *American Journal of Physiology - Renal Physiology.* **299** (1), F280-F283, (2010).

Figure1

Click here to download Figure fig1.eps

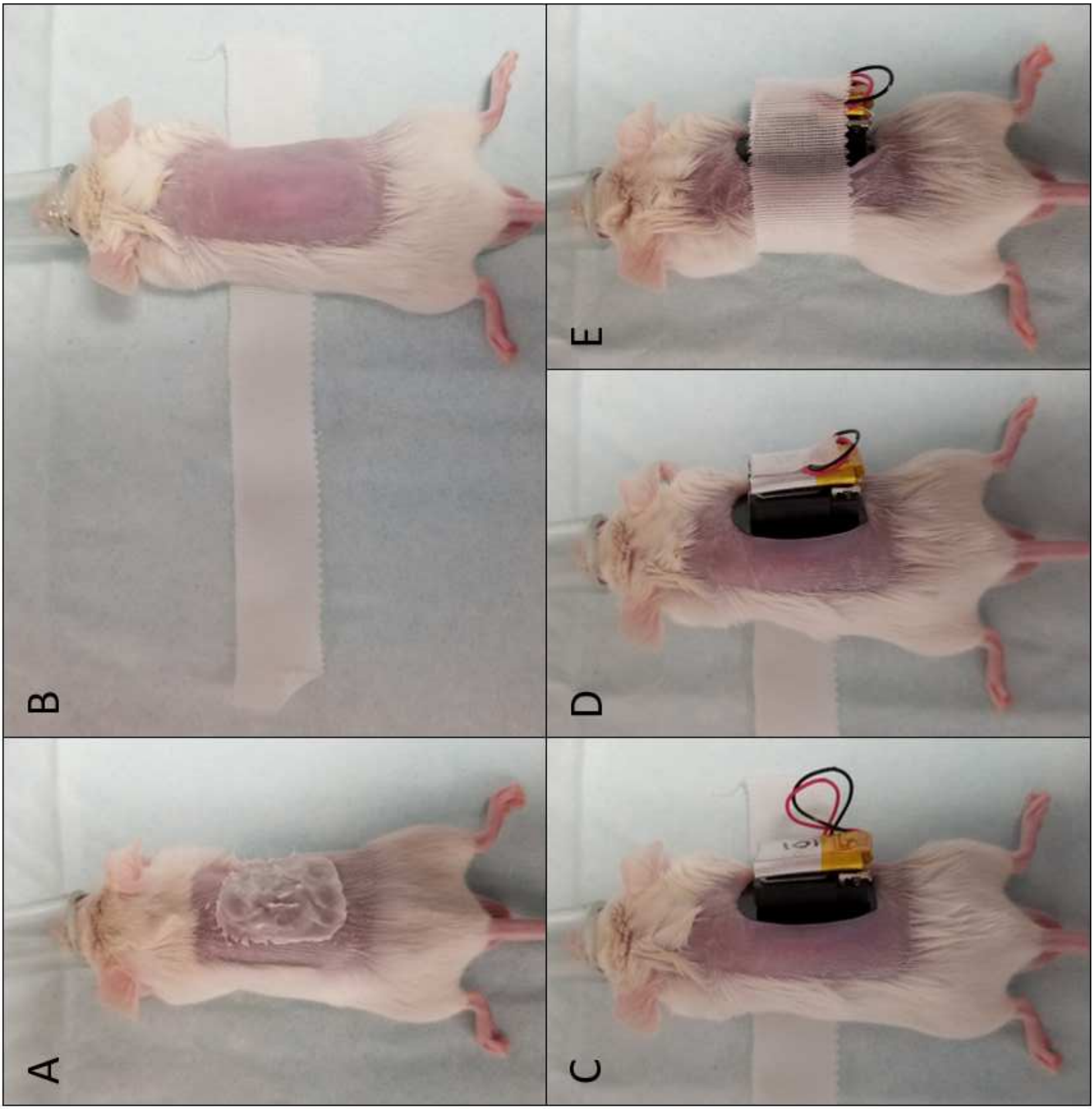
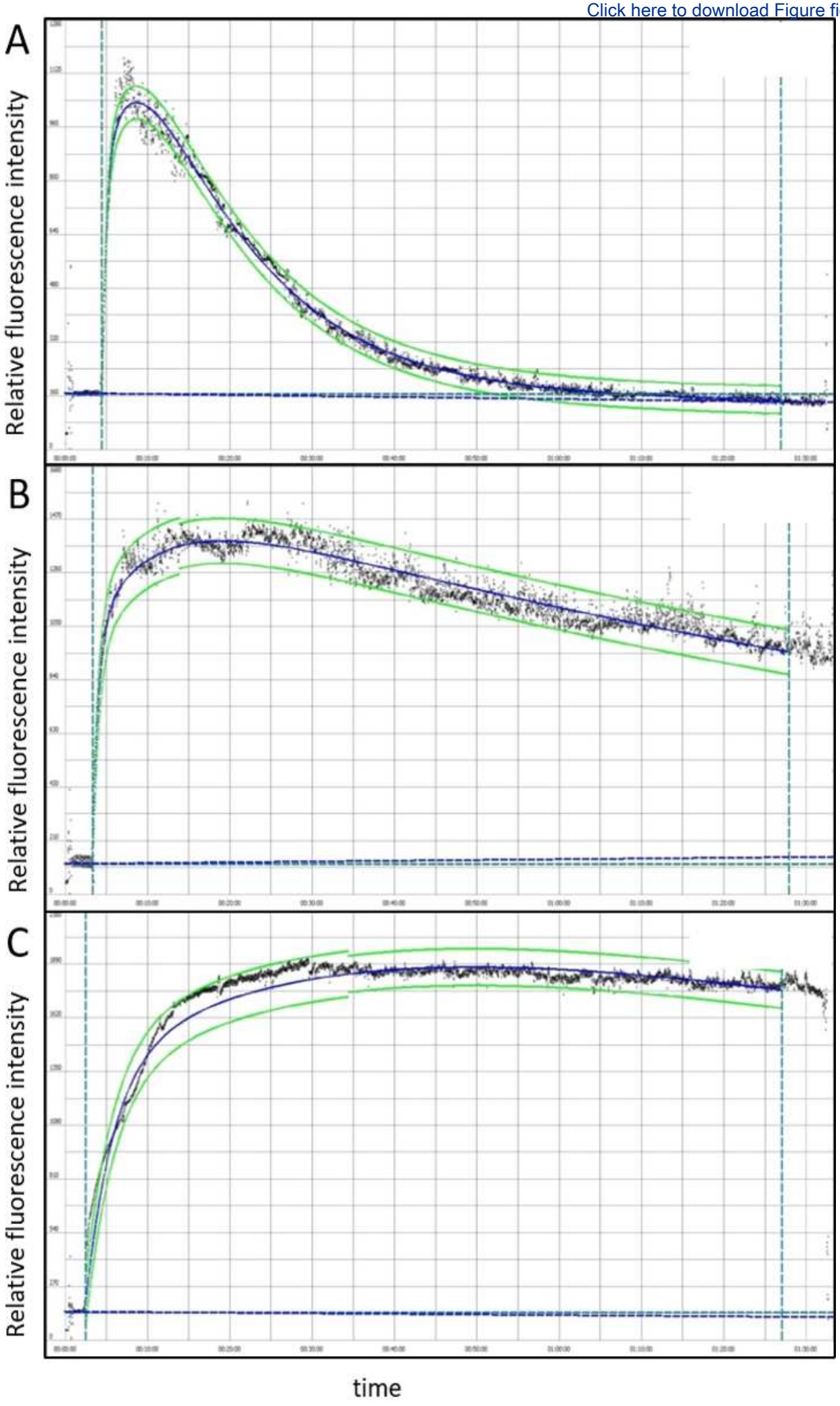


Figure2

Click here to download Figure fig2 (2).eps



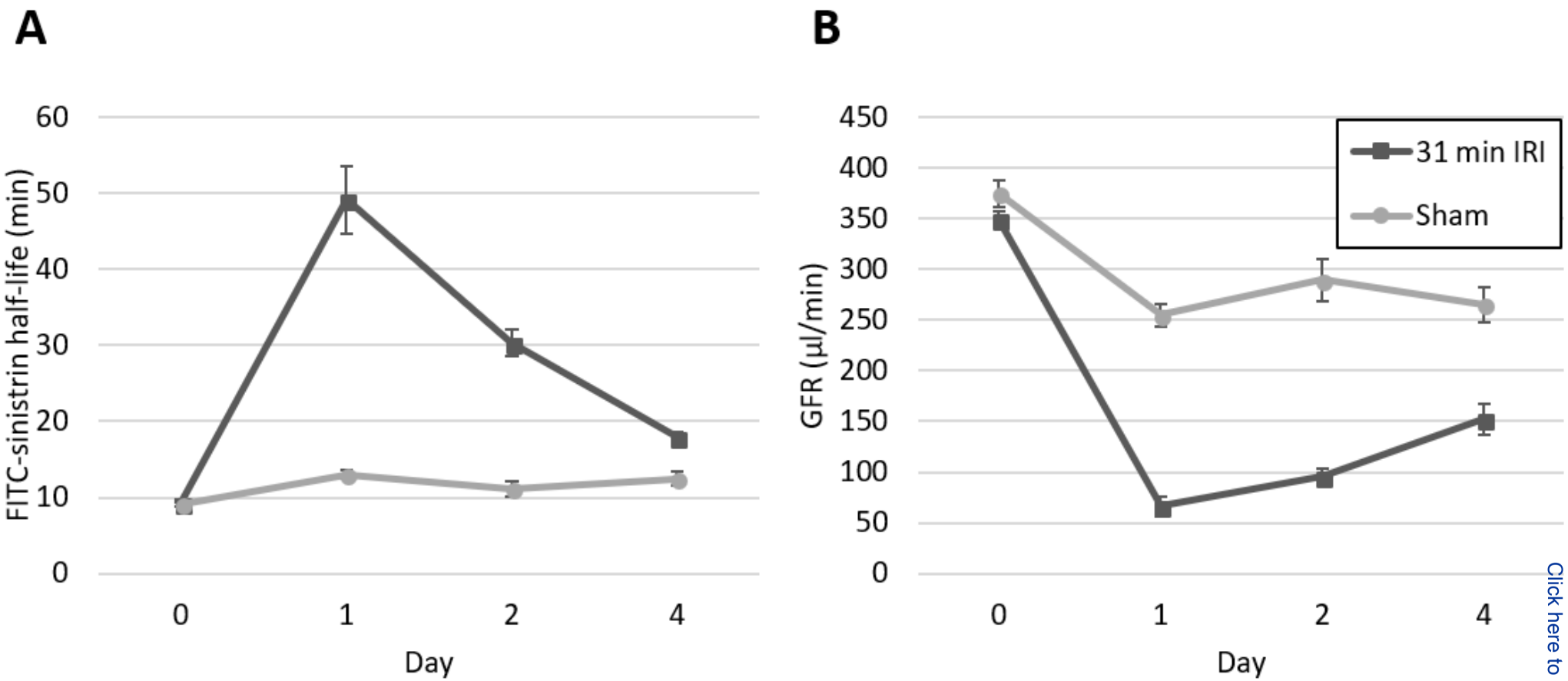


Figure4

Click here to download Figure fig4 (2).eps

Figure

Click here to download Figure Figure 5 (2).eps

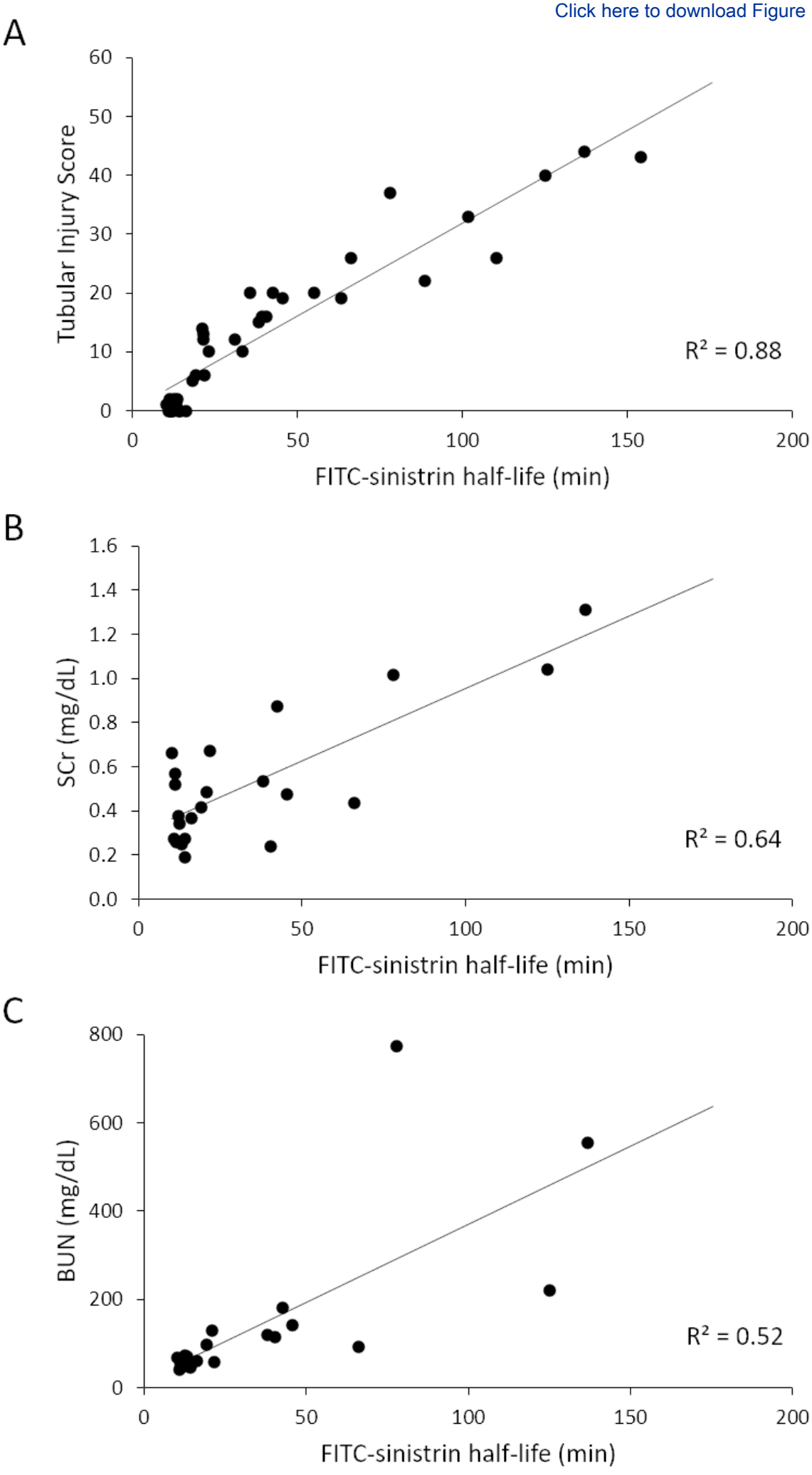


Table of Materials

Click here to download Table of Materials
Table_of_Materials.xlsx

Name	Company
Transdermal GFR monitor (comes with 1 device, 2 batteries and 1 charger)	MediBeacon GmbH
Additional Batteries	MediBeacon GmBH
Attachment patches	MediBeacon GmbH
FITC-sinistrin	MediBeacon GmbH
Hypoallergenic silk tape	e.g. Durapore (1538-2), or Kendall (7138C), or Leukosilk (01032-00)
Anaesthesia chamber, isoflurane, oxygen	
Heat pad	
Electric shaver	
Depilatory (hair removal) cream	e.g. Veet or Nair
Cotton buds	
Cotton swabs	
Timer	
Scales	
70% ethanol wipes	

Catalog Number	Comments
TDM-MH001	Reading software: MPD Lab; Analysis software: MPD Studio
PWR-BT0001	
small: PTC-SM001; large: PTC-LG001	
FTC-FS001	

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Transdermal measurement of glomerular filtration rate in mice

Author(s): Lauren Scarfe, Daniel Schock-Kusch, Lorenzo Ressel, Jochen Friedemann, Yong Shulhan Patricia Murray, Bettina Wilm, Mark de Caestecker

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☐ Standard Access ☒ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Daniel Schock-Kusch

Department:

Institution: MediBeacon GmbH

Article Title: Transdermal measurement of glomerular filtration rate in mice

Signature: [signature] Date: 23.05.2018

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Dear Editor in Chief,
Thank you for giving us the opportunity to revise our manuscript. Yours and the reviewers comments definitely helped to describe the technique more clear.
We addressed all comments below and in the manuscript.
With Kind regards,
Daniel Schock-Kusch

Editorial comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please label/number the institutional affiliation of each author sequentially. Lorenzo Ressel should be followed by 3 not 4.

This has been corrected.

3. Please revise the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

This has been completed.

4. Please define all abbreviations before use.

This has been completed.

5. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

This has been completed.

6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

This has been completed.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

This has been included.

8. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: MediBeacon GmbH, Fresenius Kabi, Kendall, Leukosilk, Veet, Nair, etc.

This has been completed.

9. 1.1-1.9: The Protocol should contain only action items that direct the reader to do something. Please move the material and equipment information to the Materials Table.

This has been completed.

10. 2.1: How many times are considered a few times?

This has been added.

11. 2.2.1: Please specify the sex, age, and strain of mouse.

This has been added to the figure legends where relevant, but not to the protocol section since different strains of mice can be used for this procedure. This is also discussed in the introduction and discussion sections.

12. Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

13. 2.2.2, 2.2.3, 4.1, 4.5, 6.4.1, 6.4.2, 6.7, 7.3, 8.1, 8.5, etc.: Please write in the imperative tense in complete sentences.
The protocol has been edited to the imperative tense.
14. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
More information has been added to the protocol
15. 4.1: Please describe how.
See response below
16. 4.3: Is this step repetitive to step 4.1?
Yes, step 4.1 was intended to introduce the hair removal steps. This has been edited for clarity.
17. 4.2, 6.1: Please mention the concentration of isoflurane and how proper anesthetization is confirmed. Are the mice anesthetized both during shaving and placement of the transdermal GFR monitor? Please clarify.
This information has been added.
18. A schematic showing the attachment of the device on the animal's back may be helpful.
We have added some photographs showing the shaving and device placement steps (new Fig 1)
19. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
This has been completed.
20. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
This has been completed.
21. Representative Results: Please elaborate the Figures more.
We have elaborated more about the figures in the text.
22. Figures 2 and 3: Please define the error bars in the figure legend.
This information has been included.
23. Figure legends: Please shorten the figure legends of Figures 1 and 4. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.
The figure legends have been shortened and some information has been moved to the main text.
24. Discussion: Please also discuss any limitations of the technique.
More information on the limitations of the technique has been added to the discussion.
25. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
This has been included now.

Reviewers' comments

Reviewer #1

Major Concerns

198-200: I cannot agree to this point. Our experience shows, that „tight" is not beneficial at all. Rather the tape has to be attached firmly to the skin/ fur. For us it is absolute imperative to not achieve firm attachment by wrapping the tape around the torso tightly (which might be intuitive to do). The adhesiveness and fixation of the imager is not a function of the tightness of the tape-wrap. We try to press the tape to the skin/fur and thereby strictly following the the skin's circumference. In our experience the mice react very sensitive to even seemingly minor restrictions around the chest. Only a mere adhesive-mediated attachment without tightening the device around the animal results in "homeostatic" conditions during the recording period, without altered and hectic moving patterns, most likely resulting in blood pressure artifacts. Also, we advise the mobilization of the upper limbs and shoulders after mounting the stripes, by gently stretching the upper limbs of the animal. This usually results in a slight (though to the animal significant) re-positioning of the adhesive stripes on the torso, which later grants more freedom of movement (in the sense of less restriction).
We thank the reviewer for his/her comments on this point and agree that the tape should not be wrapped tightly around the animal. We have re-worded the instructions accordingly to avoid misunderstanding.

249-252: Figure 2 seems redundant in light of figure 3. I would recommend to rather translate the t ½ values of figure 3 to GFR as well and show both parameters together, as done for fig 2.
We disagree that this figure is redundant, as we are showing two very different time points in IRI injury – the new figure 3 shows early, acute injury, and figure 4 shows late, chronic injury. We have now edited the figures and legends to make the difference clear, and we have also switched figures 3 and 4 so that the acute data is shown before the chronic data. Additionally, we have included both half-life and GFR graphs for both figures.

258-260: Although we use semi-quantitative tubular injury scoring ourselves, the method lacks sensitivity (and reliability). The by far most sensitive parameter of kidney injury is protein expression or mRNA transcription of Havcr1 (aka Tim1, Kim1). A good correlation with this parameter would strengthen the point, that transcutaneous GFR measurement is in fact reflecting kidney health.
We would like to thank the reviewer for this comment, however while we agree that Kim1 mRNA and protein are considered valuable markers of renal injury, many (including ourselves) still believe that histopathology is the gold standard for assessing kidney damage. While we accept that the two assays are complementary, we are not aware of any publication which suggests that Kim-1 supersedes the value of histopathology to assess the severity of renal injury. Furthermore, papers that evaluate Kim-1 still use histopathology as their gold standard, for example https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2744478/. The reviewer should note that histopathology scoring was performed by a qualified ECVP Board Certified Veterinary pathologist who was blinded to the treatment of the animal, and as such we have confidence that the scoring is as objective and as accurate as possible.

On the contrary, discussing the supremacy of GFR over SCr and BUN (poorer correlation) is pointless, since the field is well aware of the fact, that both parameters are poor biomarkers for

kidney function and health. Their predominance in the field is only due to cost benefits in the clinic. On the contrary, investigating the correlation of transdermal GFR assessment and Scr (Jaffe reaction and/ or HPLC) and BUN, respectively, would be more interesting. In my opinion, this would bring greater impact to the authors manuscript, as well, since Scr and BUN are still widely used in preclinical settings, where GFR would be way more appropriate (and available thanks to the authors magnificent invention). Showing correlations of t ½ vs Scr and t ½ vs BUN will point out, that those biomarkers are obsolete, especially in translational kidney research.
This is a very good point and we thank the reviewer for suggesting this. We have changed the correlation graphs such that tubular injury, serum creatinine and BUN are each correlated with FITC-sinistrin half-life and include discussion about this in the manuscript.

307-317: The point made regarding dermal pigmentation and measurement sensitivity is indeed a FAQ and is very well addressed. Nevertheless, the complete lack of a (short) comparison with other available methods to measure GFR is surprising and weakens the manuscript's potential impact.
We have now included a short comparison of pros and cons of the technique to other methods.

Minor Concerns

160-161: The patch size is not specified beforehand and a cut off is only meaningful if rat-sized patches are used. The smaller (round-edged) patches available from MediBeacon fit perfectly for mice. Here, further cut off is not beneficial. Inside-out-folded adhesive stripes can be used to attach the battery to the device, if no excess patch material is available (as done in our lab without any problems).
We have now edited the text to describe both types of patches.

169-191: For operators with narcosis chambers I would suggest the following approach: First, assemble the device, attach the battery, check if it's working and remove the backing from the adhesive patch. Put it upside down on the table to keep it ready. Ready the adhesive tape by laying it out adhesive side up on the table next to the device. Only then anesthetize the animal. Upon removal of the mouse from the chamber, wipe the skin with ethanol and dry quickly with another cotton swab. Attach the fully assembled imager to the mouse before moving mouse and imager to the adhesive tape. Fixate the tape to the mouse. This approach proved to be extremely fast by simultaneously greatly reducing the anesthesia time (and hence depth of anesthesia and occurrence of associated artefacts).
We thank the reviewer for this description of their method, however we do not agree that this is a significantly faster way of performing the protocol. Our protocol instructs to prepare the device, anaesthetize the mouse, attach the battery, then attach the device, so the only difference to the protocol described by the reviewer above is when the battery is attached. It only takes a few seconds to attach the battery so doing this before anaesthetizing the mouse does not reduce overall anesthesia time.

193-196: Left-right confusion? 194: "right" instead of "left"?
We thank the reviewer for pointing out this mistake, and it has now been corrected. Further information has also been added to clarify the right/left instructions.

221-226: We advise beginners to utilize isoflurane narcosis for this step as well. This is in favor of prolonged battery survival.
We have included an alternative step to describe anesthetizing the mouse for device removal, however we would like to point out that this does not affect battery survival. In fact it will actually take longer to remove the device the mouse, as you have to wait for it to fall asleep and in this time the mouse will be jumping around the chamber and could get a paw stuck in the battery wires, thus possibly damaging the wires. Also, the second anesthesia session in a short space of time may affect the wellbeing of the mouse, especially if it is already suffering from kidney injury. We have taught a number of beginners to remove the device on a conscious mouse and it is not very difficult.

Material table examples incomplete. A complete list might be of great interest for establishing the method anew.
We have now included this.

Low picture quality.
We have now included higher quality images.

Reviewer #2

Minor Concerns
The reviewer has few suggestions, which are mostly minor nature that will potentially improve this work:

Introduction part lacks general aim of the presented manuscript. Adding a sentence providing rational for the need for described protocol would be helpful for the reader to follow the story.
Additional information has been added at the end of the introduction.

Protocol, part 4 (remove hair from the mouse) - what if the skin (after removing hair) appears irritated or wounded? Authors should consider adding a sentence commenting on the condition of the skin.
Additional information has been added to address this point.

Protocol, part 4 (FITC-Sinistrin injection) - Author need carefully describe the light sensitivity of prepared injection solution.
This is already addressed in the protocol in step 1.1 – lines 126-127 of the original version, lines 109-110 of the new version.

Representative results - Authors present GFR results obtained from BALB/c mice. What about other commonly used mouse strains (C57BL6 or 129)? Addition of a comment about other stains would be helpful for potential readers.
We have included a comment on this and referred to a list of publications.

-Representative results - Authors may consider adding a table with reference/control values for GFR in mice. That would greatly help researchers to interpret their data.

We agree with the reviewer that such a table would be helpful to the reader, however it is difficult to provide reference values that will account for all of the variabilities in mice (age, gender, strain etc.). Furthermore, we have already provided the readers with a small amount of reference values in figures 2 and 3 (fig 3 and 4 in new version). We have now included some more references to direct the reader to published literature for additional data.

Reviewer #3

Minor Concerns

1) In item 6.8 in the protocol, the authors suggest collecting a background reading in anesthetized mice. Considering that a correct baseline reading is critical to obtain an accurate GFR measurement, is it possible the anesthesia might modify the level of this baseline reading? Although acquiring a baseline reading in conscious animals will probably have more artifacts, it might be more accurate and representative of the actual GFR measurement.
We thank the reviewer for this suggestion, however it is not possible to wake the animal up for the baseline measurement. This is because of the restraint that would be required to perform an IV tail injection in a conscious mouse – the mouse won't fit in the restrainer with the device attached, and the pressure on the device during restraint would disrupt the readings. Additionally, a new algorithm has been developed to correct for these shifts in the baseline reading, this is now mentioned in the discussion.

2) For Figures 3 and 4, authors should include the actual GFR value besides the FITC-sinistrin half-life as it is more physiologically relevant.
This has now been included for Figures 3 and 4. Figure 5 still uses FITC sinistrin half-life for clarity of presentation, since half-life is directly proportional to the severity of injury, while GFR is inversely correlated with injury.

3) For Figure 4, it would be interesting to include a correction GFR vs. BUN or GFR vs. SCr as they are directly related.
This is an excellent idea, and has now been changed per the reviewer's suggestion.