Am J Physiol Renal Physiol 303: F783–F788, 2012.
First published June 13, 2012; doi:10.1152/ajprenal.00279.2012.

Transcutaneous measurement of renal function in conscious mice

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Submitted 15 May 2012; accepted in final form 11 June 2012

Schreiber A, Shulhevich Y, Geraci S, Hesser J, Stsepankou D, Neudecker S, Koenig S, Heinrich R, Hoecklin F, Pill J, Friedemann J, Schweda F, Gretz N, Schock-Kusch D. Transcutaneous measurement of renal function in conscious mice. *Am J Physiol Renal Physiol* 303: F783–F788, 2012. First published June 13, 2012; doi:10.1152/ajprenal.00279.2012.—Determination of glomerular filtration rate (GFR) in conscious mice is cumbersome for the experimenter and stressful for the animals. Here we report on a simple new technique allowing the transcutaneous measurement of GFR in conscious mice. This approach extends our previously developed technique for rats to mice. The technique relies on a miniaturized device equipped with an internal memory that permits the transcutaneous measurement of the elimination kinetics of the fluorescent renal marker FITC-sinistrin. This device is described and validated compared with FITC-sinistrin plasma clearance in healthy, unilaterally nephrectomized and *pcy* mice. In summary, we describe a technique allowing the measurement of renal function in freely moving mice independent of blood or urine sampling as well as of laboratory assays.

fluorescein isothiocyanate-sinistrin; kidney function; glomerular filtration rate; transcutaneous; fluorometry

THE DETERMINATION AND MONITORING of glomerular filtration rate (GFR) are essential when phenotyping animal models or assessing toxicity of novel chemical or medical agents. Especially in mice the measurement of GFR using bolus or constant infusion clearance techniques is difficult and cumbersome (14). For blood sampling during bolus clearance experiments for instance, the tail or saphenous vein has to be punctured seven times within 75 min (4, 14). This procedure is stressful for conscious animals, as they have to be restrained repeatedly. Blood sampling without anesthesia, however, is essential to avoid an anesthesia-related decrease in GFR (2, 6).

Plasma creatinine is often used as surrogate marker of renal function. Recent findings, however, revealed that ~50% of the excreted creatinine of male mice and ~35% of the excreted creatinine of female mice is secreted by the tubules rather than filtered by the glomeruli (4). Due to these findings and problems related to creatinine assays (7), plasma creatinine has to be regarded as a poor marker of renal function in mice.

Several approaches for a transcutaneous measurement of GFR have been developed (3, 10–13, 15–19, 21). All of them allow the measurement of GFR without blood and/or urine sampling and are based on the elimination kinetics of fluorescent exogenous markers. None of these approaches, however, allows measurements in conscious mice.

Here we describe a method permitting the measurement of GFR in conscious mice. This approach is an extension of our previously published method used in rats (19). The new technique is based on a miniaturized device equipped with an internal memory allowing the transcutaneous measurement of the elimination kinetics of the fluorescent renal marker FITC-sinistrin (10–12, 17–19). This device is described, and the validity of the transcutaneous compared with the plasma clearance is proven in healthy, unilaterally nephrectomized (UNX) and *pcy* mice (20).

MATERIALS AND METHODS

Device. The miniaturized instrument (Fig. 1) is built up from two light-emitting diodes with an emission maximum for FITC at 470 nm (KPTD-3216QBC Kingbright Electric) and a photodiode detecting the fluorescent light with a maximum sensitivity at 525 nm (EPD-525–1-0.9–1; EPIGAP Optoelektronik; Fig. 1). To select the appropriate wavelength, three filter layers are placed in front of the photodiode (767 Oklahoma Yellow; >500 nm; Lee Filters). After highly sensitive amplification and digitization (16 bit), the data are stored in the internal memory of the microcontroller (MSP430f2013 Texas Instruments; maximum storage capacity 256 data points). After the measurement the data set is downloaded to a PC (via USB), where the kinetic parameters are determined. For use in conscious mice, the energy is supplied by a small lithium polymer rechargeable battery.

FITC-sinistrin. Sinistrin is the active pharmaceutical ingredient of the commercially available GFR marker Inutest (Fresenius Kabi, Linz, Austria) used in human and veterinary medicine. The labeling reaction has been described elsewhere (10–12) By improving the control of the chemical synthesis, the fluorescent yield of the FITC-sinistrin (Mannheim Pharma & Diagnostics, Mannheim, Germany) could be increased by a factor of four compared with previous studies (17, 19). FITC-sinistrin is highly soluble in aqueous solvents at room temperature. Moreover, no dialysis of the substance before the experiment is necessary to eliminate unbound FITC as described for FITC-inulin (14) (for additional data about the purity of FITC-sinistrin see APPENDIX).

Proof of principle. For validation, bolus clearance experiments were performed in conscious mice (C57Bl/6–129 SV; $n = 8$), prior and 3–4 days after UNX ($n = 8$) and *pcy* (20) mice (nephronophthisis; $n = 6$). All animal experiments were approved by the local ethics

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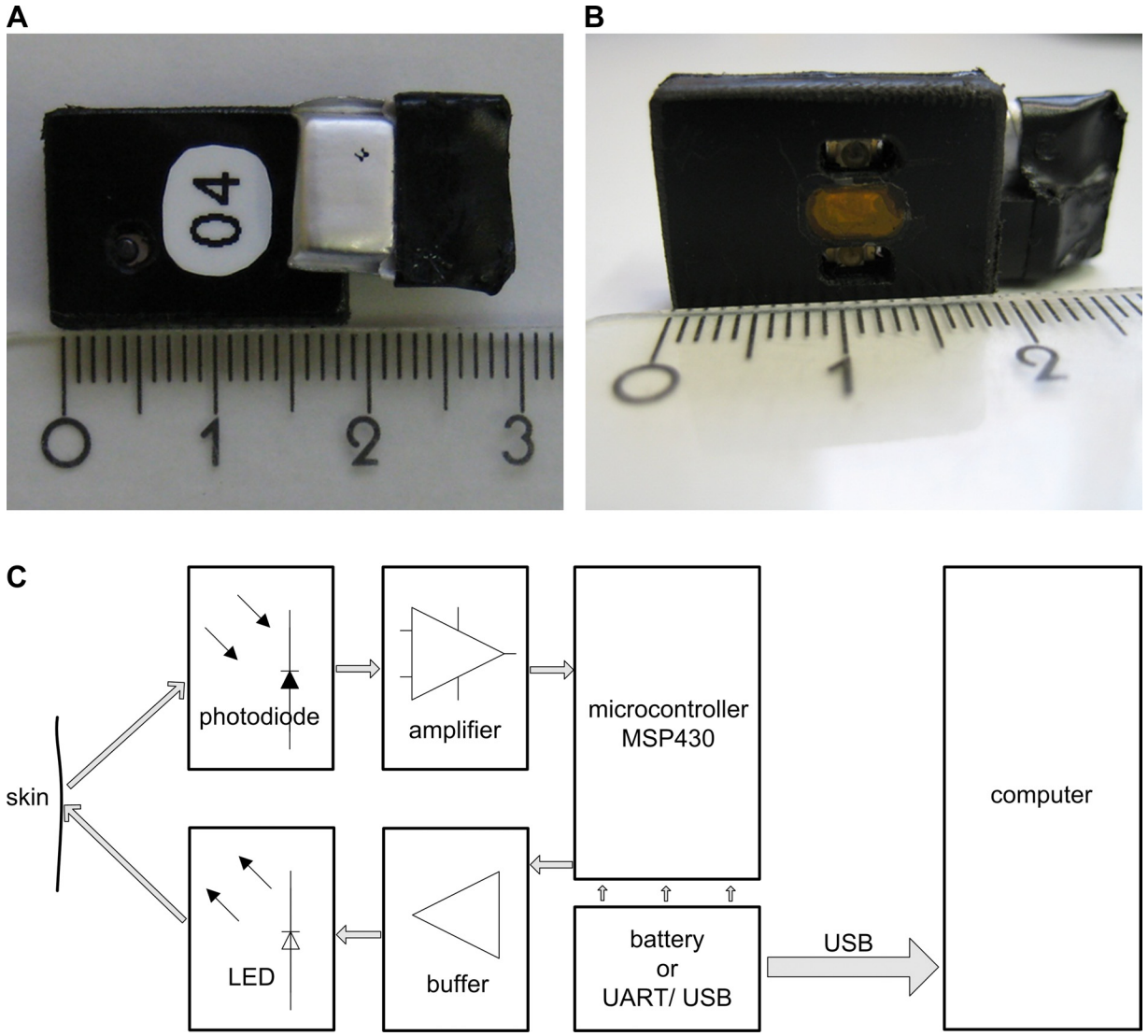


Fig. 1. Top (*A*) and bottom (*B*) view of the miniaturized device with battery for transcutaneous measurement (ruler in cm). *C*: schematic drawing of its building blocks.

committees (Regierungspräsidium) responsible for the participating institutions.

To compare both the transcutaneous and the plasma measurements, the conscious mice have to be restrained seven times for the blood sampling (4, 14). In the restrainer, a transcutaneous measurement was not possible, as the device would have been pressed against the wall of the restrainer resulting in signal artifacts. Therefore, the GFR measurements with the two techniques were performed on consecutive days in the same animals.

Plasma clearance. While the animals were under short isoflurane (Abbott Laboratories, Abbott Park, IL), anesthesia FITC-sinistrin (5.6 mg/100 g body wt dissolved in NaCl 0.9%) was injected. Blood samples (~5 μl) were collected by puncturing the tail vein 3, 7, 10, 15, 35, 55, and 75 min after injection. The measurements in plasma were performed for 75 min to replicate established protocols (4, 14). The mice were repeatedly placed into a restraining tube for each blood sample. The fluorometric measurement was conducted using a Nanoquant plate (Tecan Group, Männedorf, Switzerland) and a fluorospectrometer (Tecan Group) at an excitation wavelength of 470 nm and an emission wavelength of 520 nm. For the measurement, the plasma was diluted with 500 mM HEPES (pH 7.4). Quantification was achieved by using FITC-sinistrin standard curves.

Transcutaneous bolus clearance. While the animals were under isoflurane anesthesia a part of the back of the mice was depilated. Afterwards the device was fixed on this region using a double-sided adhesive patch (Lohmann, Neuwied, Germany). The device was started to measure the background signal for 1 min before the FITC-sinistrin (15 mg/100 g body wt dissolved in NaCl 0.9%) was injected. Measurement time was 60 min with a measurement interval of ~14 s. In respect to signal-to-noise ratio, the transcutaneous measurement was stopped after 60 min.

GFR calculation. GFR was calculated from FITC-sinistrin plasma clearance using an established two-compartment model (4). Transcutaneous GFR was calculated using the half-life derived from the rate constant (α_2) of the slow, single exponential; excretion phase of the excretion curve (Fig. 2) and a semiempirical conversion factor (17). The mouse specific conversion factor was established using an equal procedure as previously described for rats, however, using two-compartment plasma GFR values as basis (17). This leads to a reduced conversion factor, reflecting estimated distribution volume, compared with the factor described for rats (17). For plasma clearance, GFR was also calculated from the half-life derived of the slow, single exponential excretion phase from the two-compartment model and the mouse specific conversion factor.

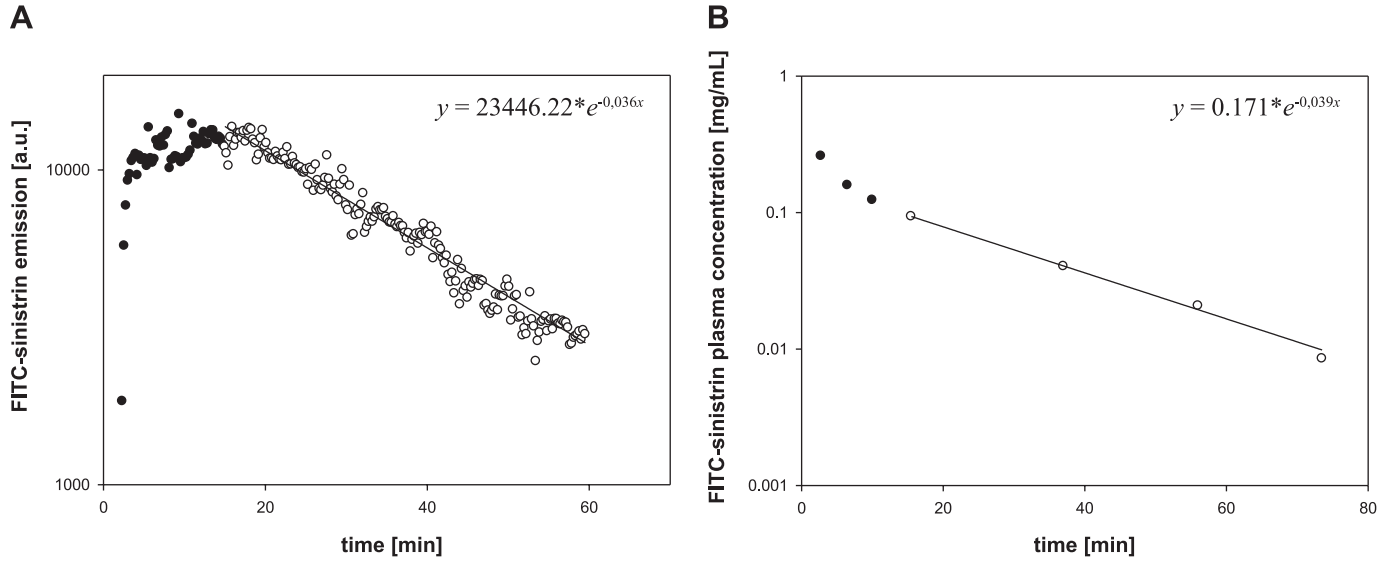


Fig. 2. Semilogarithmic plots of fluorescence signals measured transcutaneously and in plasma after FITC-sinistrin application in mice. A typical example of a transcutaneously measured FITC-sinistrin kinetic with its characteristic rising phase (●) followed by a single exponential decreasing phase (○) is depicted in *A* (17, 19). Black solid line represents the single exponential fitting with its slope α_2 (excretion rate). Corresponding kinetic measured in plasma is given in *B*. A fast initial distribution phase (●) is followed by a characteristic excretion kinetic. Exponential regression curve is fitted to the slow, single exponentially decreasing part of the curve (solid line) with its slope α_2 (○). Semilogarithmic plotted plasma excretion kinetic shows an switch into the single exponential renal related excretion phase ~15 min after FITC-sinistrin injection. Therefore, the single exponential fit for the transcutaneous assessment of glomerular filtration rate (GFR) was also started 15 min after FITC-sinistrin bolus injection.

$$\mathrm{GFR}\left[\mu\mathrm{L}\cdot\mathrm{min}\cdot 100\ \mathrm{g\ bw}\right] = \frac{14616.8\left[\mu\mathrm{L}/100\ \mathrm{g\ bw}\right]}{t_{1/2}(\mathrm{FITC-sinistrin})[\mathrm{min}]} \quad (1)$$

Figure 2 shows representative excretion kinetics determined transcutaneously as well as fluorometrically in plasma samples.

Urinary recovery of FITC-sinistrin in mice. Recovery of FITC-Sinistrin in urine has been shown in rats previously (10). To ensure urinary excretion in mice, recovery experiments were performed in C57BL/6 mice ($n = 3$). The animals were anesthetized (Sevofluran, Baxter, Deerfield IL), the urinary bladder was catheterized, and FITC-sinistrin was injected. Urine samples were collected via the catheter at time points 5, 10, 15, 30, 45, 60, 75, and 90 min after injection. Excreted FITC-sinistrin was quantified fluorometrically after dilution of the urine 1/400 with 500 mM HEPES (pH 7.4).

RESULTS AND DISCUSSION

Urinary recovery of FITC-Sinistrin in mice. Urinary recovery of FITC-sinistrin in the three examined mice was 101.1 ± 2.3% of the injected dose. Complete urinary recovery of FITC-sinistrin indicates exclusive renal excretion.

Method comparison of GFR measurements obtained transcutaneously and in plasma. The results of the GFR measurements assessed transcutaneously and in plasma in three animal models are summarized in Table 1.

The mean GFR values of the plasma clearances and the transcutaneously assessed GFR are highly comparable in the three animal groups. Also, the GFR values assessed 3–4 days after UNX with ~70% of the baseline values are reasonable. A recovery of GFR to ~70% of baseline 3–4 days after UNX is also documented for rats (5). The results are even more convincing as similar results are obtained with the three calculation methods.

In Fig. 3, direct comparisons of the transcutaneous and the plasma clearance based GFR measurements of the single animals are depicted.

In Fig. 3*A*, major differences between the transcutaneous and the two-compartment plasma clearance GFR in some animals can be noted. In part, this discrepancy can be assigned to a day-to-day variability of the GFR and the more robust curve fitting procedure using the transcutaneous method (19). Another factor contributing to the weak correlation might be the strong dose dependence of the classical plasma clearance techniques as GFR = dose/area under curve (9). In an one-compartment GFR assessment, area under curve is determined as *y*-axis intercept of the extrapolated single exponential slow decay part divided by α_2. For this reason, a linear behavior between α_2 and one-compartment plasma clearance GFR is expected, if the volume of distribution of the animals is comparable. However, as seen in Fig. 3*B*, the one-compartment

Table 1. *Results of the GFR assessments in the three mouse models using the transcutaneous, the two-compartment plasma clearance, and the one-compartment plasma slope only method combined with Eq. 1*

	GFR, $\mu l\cdot min^{-1}\cdot 100$ g body wt^{-1}		
Mouse Model	Transcutaneous	Two-Compartment Plasma	One-Compartment Plasma (*Eq. 1*)
Healthy ($n = 8$)	1,381 ± 264	1,373 ± 182	1,212 ± 274
UNX ($n = 8$)	943 ± 189	938 ± 194	883 ± 87
pcy ($n = 6$)	713 ± 207	681 ± 308	712 ± 84

Values are means ± SD. GFR, glomerular filtration rate; healthy and unilaterally nephrectomized (UNX), C57Bl/6–129 SV mice; *pcy*, mouse model of nephronophthisis (20).

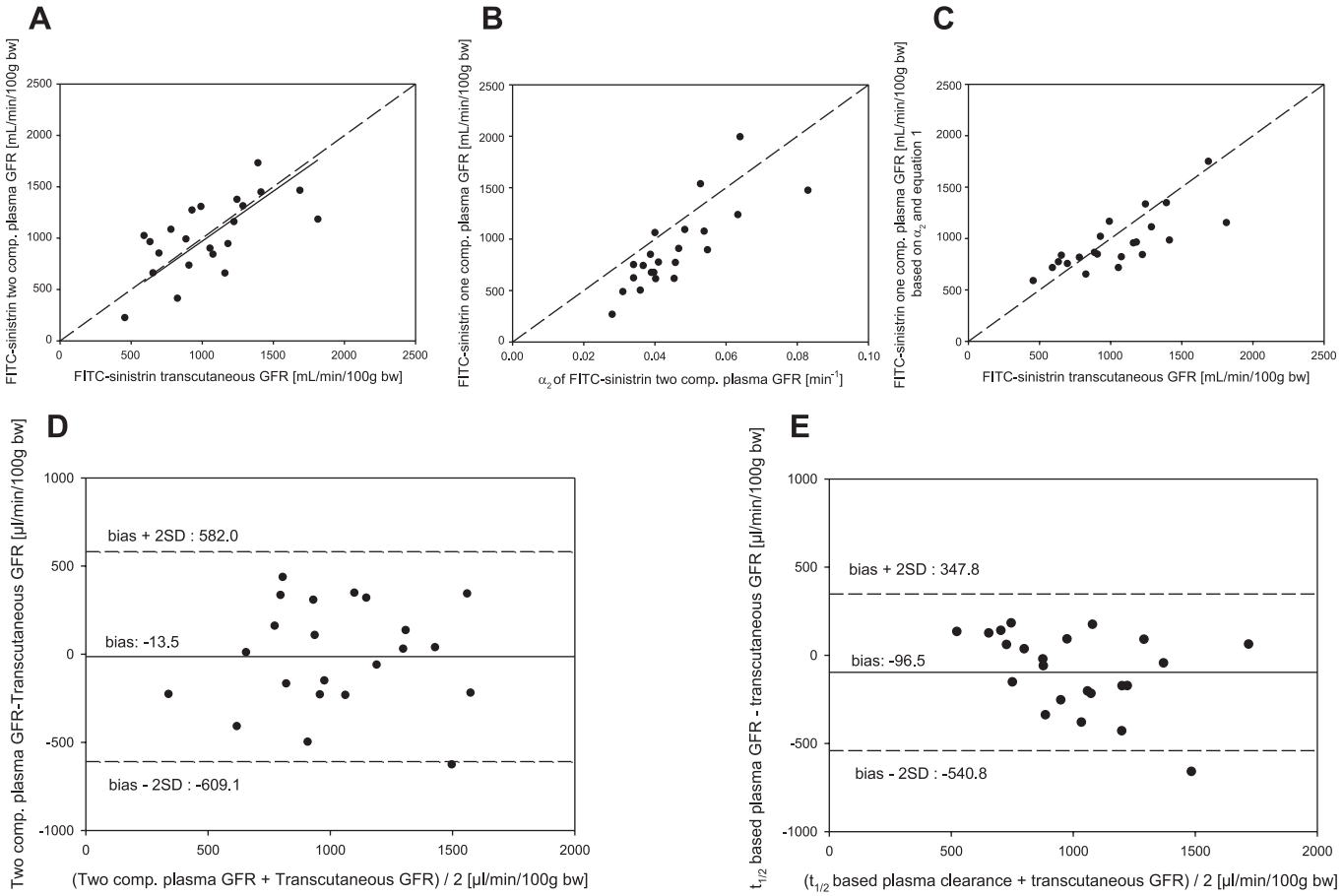


Fig. 3. *A*: direct comparison of the GFR values measured on consecutive days with the transcutaneous and the classical two-compartment plasma clearance method (dashed line: line of identity; solid line: linear regression with $r^2 = 0.33$). *B*: comparison of the rate constant α_2 of the slow, single exponential decay of the two-compartment plasma fitting curve vs. a classical one-compartment slope-intercept GFR method (dashed line: line of identity; solid line: linear regression with $r^2 = 0.33$). *C*: comparison of the transcutaneous GFR and the plasma GFR assessed by $t_{1/2}$ using the introduced conversion factor for both methods (dashed line: line of identity; solid line: linear regression with $r^2 = 0.42$). *D*: Bland-Altman plot of the two-compartment plasma GFR and the transcutaneously assessed GFR. *E*: Bland-Altman plot of the plasma GFR assessed by $t_{1/2}$ using the introduced conversion factor and transcutaneously assessed GFR.

plasma-clearance GFR values can vary tremendously at virtually equal α_2 values. The dose dependence is especially pronounced in mouse experiments, as extremely small volumes (< 0.1 ml) have to be injected. Thus minor errors in the injected volume or concentration of the injected fluid have a huge impact on GFR. By using the same approach for plasma GFR assessment as used for the transcutaneous measurement based on $t_{1/2}$ and the introduced conversion factor, this error can be reduced as shown in Fig. 3*C* and the narrower 95% limit of agreement in the Bland-Altman plot shown in Fig. 3*E* compared with Fig. 3*D*.

The theoretical background of rate constant-based GFR assessment and the transcutaneous measurement is beyond the scope of this technical note; the interested reader is referred to recent literature (1, 9, 17, 19).

The transcutaneous measurement was tolerated well by all animals. After the recovery phase from the isoflurane anesthesia (present in both approaches), no impact of the device on the movement or behavior of the mice was observed. This observation indicates that the stress level of the mice is lower compared with the classical plasma clearance methods requiring repeated restraining and vessel puncture.

The strong movements of the mice during the recovery from excitation phase of isoflurane anesthesia led to pressure-related measurement artifacts during the first 1–3 min of the transcutaneous measurement. This made a two-compartment fit of the transcutaneously assessed data as published for rats impossible (19). Therefore, a one-compartment model for the transcutaneous GFR assessment was applied (17). As with the transcutaneous approach no tail vein punctures are needed, repetitive GFR measurements can be performed within a short period of time in the same animal. Moreover, fibrotic changes and vessel stenosis/obstruction due to repeated punctures are avoided (8).

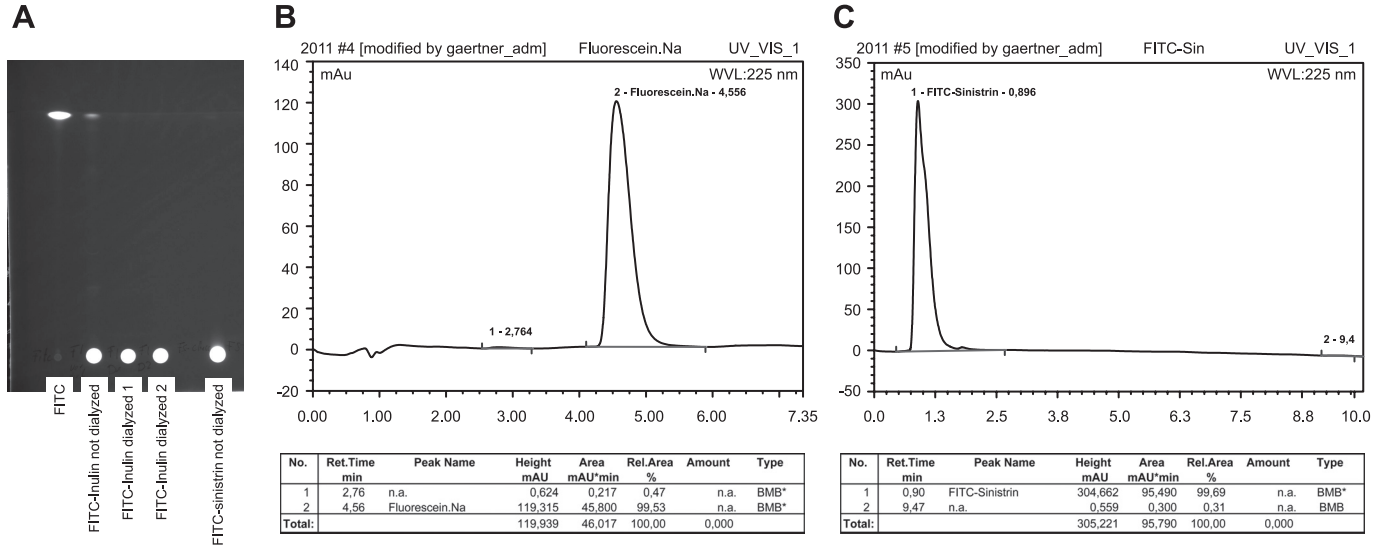
In summary, we present a new technique allowing the determination of renal function in freely moving mice without blood or urine sampling as well as without laboratory assays. The technique is validated against a classical plasma clearance in healthy, UNX, and *pcy* mice.

APPENDIX: PURITY OF FITC-SINISTRIN

The purity of FITC-Sinistrin in respect of unbound FITC was checked by thin layer chromatography as well as by high-pressure liquid chromatography (Fig. *A1*).

ACKNOWLEDGMENTS

The excellent technical assistance of Dr. Bettina Kraenzlin, Elisabeth Wuehl, and Viktoria Skude, is gratefully acknowledged. Fresenius Medical



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	2,76	n.a.	0,624	0,217	0,47	n.a.	BMB*
2	4,56	Fluorescein.Na	119,315	45,800	99,53	n.a.	BMB*
Total:			119,939	46,017	100,00	0,000	

No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0,90	FITC-Sinistrin	304,662	95,490	99,69	n.a.	BMB*
2	9,47	n.a.	0,559	0,300	0,31	n.a.	BMB
Total:			305,221	95,790	100,00	0,000	

Fig. A1. *A*: photograph of a thin layer chromatogram (TLC) illuminated with an ultraviolet (UV) lamp is shown (solid phase: silica 60; eluent: aceton/H_2O 9/1). Plate was loaded with 4 μl of 10 mg/ml solutions of the respective samples. It is clearly demonstrated, that the FITC sample runs with the mobile phase. The sample of undialyzed FITC-inulin (Sigma-Aldrich) shows contamination with unbound FITC (FITC-inulin sticks to solid phase). In contrast, the two independently dialyzed FITC-inulin samples (14) as well as the undialyzed FITC-sinistrin (sticks to solid phase) sample are apparently free of unbound FITC. The purity of the FITC-sinistrin was additionally examined by HPLC [eluent: acetonitril/KH_2PO_4 (5 mM, pH 2.0) 70/30; column: Agilent Zorbac Eclipse C8XDB; flow rate: 1 ml/min; temperature: 35°C; detection: UV 225 nm]. A run with a pure FITC sample shows a retention time of 4.56 min (*B*) whereas a FITC-sinistrin sample 0.9 min (*C*). Lack of a second peak at 4.56 min at the latter confirms the purity of the FITC-sinistrin as demonstrated by TLC.

Care provided a PhD grant (Nephrocore; to Y. Shulhevich). FITC-Sinistrin and the measuring device are products of Mannheim Pharma & Diagnostics and commercially available for research use. For further questions, please contact the corresponding author.

DISCLOSURES

D. Schock-Kusch, J. Pill, J. Hesser, and N. Gretz are inventors on patents and patent applications covering this subject, as well as founders of Mannheim Pharma & Diagnostics (distributor of FITC-sinistrin). J. Pill is Managing Director of Mannheim Pharma & Diagnostics. Y. Shulhevich was supported by a grant (nephrocore) from Fresenius Medical Care. D. Schock-Kusch was supported by EU-Framework Programme 7: PLACE-it. S. Geraci was supported by ITN Marie-Curie EU-Framework Programme 7: NephroTools.

AUTHOR CONTRIBUTIONS

Author contributions: A.S., Y.S., J.H., D.S., S.K., R.H., F.H., J.P., J.F., F.S., N.G., and D.S.-K. conception and design of research; A.S., Y.S., S.G., S.K., F.H., and D.S.-K. performed experiments; A.S., Y.S., S.G., J.H., D.S., S.N., S.K., R.H., J.P., J.F., F.S., and D.S.-K. analyzed data; A.S., Y.S., S.G., J.H., D.S., S.N., S.K., R.H., J.P., J.F., F.S., N.G., and D.S.-K. interpreted results of experiments; A.S., J.P., and D.S.-K. drafted manuscript; S.N., J.P., F.S., N.G., and D.S.-K. edited and revised manuscript; F.S. and N.G. approved final version of manuscript; D.S.-K. prepared figures.

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