Video Article

The Mouse Isolated Perfused Kidney Technique

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Abstract

The mouse isolated perfused kidney (MIPK) is a technique for keeping a mouse kidney under *ex vivo* conditions perfused and functional for 1 hr. This is a prerequisite for studying the physiology of the isolated organ and for many innovative applications that may be possible in the future, including perfusion decellularization for kidney bioengineering or the administration of anti-rejection or genome-editing drugs in high doses to prime the kidney for transplantation. During the time of the perfusion, the kidney can be manipulated, renal function can be assessed, and various pharmaceuticals administered. After the procedure, the kidney can be transplanted or processed for molecular biology, biochemical analysis, or microscopy.

This paper describes the perfusate and the surgical technique needed for the *ex vivo* perfusion of mouse kidneys. Details of the perfusion apparatus are given and data are presented showing the viability of the kidney's preparation: renal blood flow, vascular resistance, and urine data as functional, transmission electron micrographs of different nephron segments as morphological readouts, and western blots of transport proteins of different nephron segments as molecular readout.

Video Link

The video component of this article can be found at https://www.jove.com/video/54712/

Introduction

The isolated perfusion of organs has been the subject of an ongoing effort among physiologists for many decades[1]. The technique enables the function of the organ, without systemic influences such as blood pressure, hormones, or nerves, to be studied. Carl Eduard Loebell is considered to be the first to have described the successful perfusion of an isolated kidney, in 1849[2]. Since then, the perfusion apparatus has undergone significant refinement. Frey and Gruber introduced an artificial lung for oxygenation and pulsatile pumps for continuous perfusion[2]. While early researchers mainly studied the kidneys of large mammals-namely, pigs[2] and dogs[3]-the first report of the use of rat kidneys, by Weiss *et al.*, was a milestone in the study of small-mammal-organ perfusion[4]. Schurek *et al.* reported the necessity of adding mammalian erythrocytes to the perfusate if sufficient renal tubular oxygenation was to be achieved[5]. Critical for long-term experiments was the introduction of continuous dialysis of the buffer by the same research group[6]. In 2003, Schweda *et al.* were the first to report a functional mouse isolated perfused kidney (MIPK)[7], later refined by Rahgozar *et al.*[18] and Lindell *et al.*[14].

While technically more challenging than the rat isolated perfused kidney, the use of the MIPK bears the advantage of enabling the use of a wide array of genetically altered mice. This paper presents the details of the authors' method for perfusing isolated mouse kidneys for 1 hr. The method allows for the continuous assessment of renal flow rate, vascular resistance, hormone release, blood gas analysis, urine analysis, and the application of drugs. Following the procedure, kidneys could be processed for molecular and biochemical analysis, be fixed for microscopy, or transplanted into a recipient mouse (**Figure 1**).

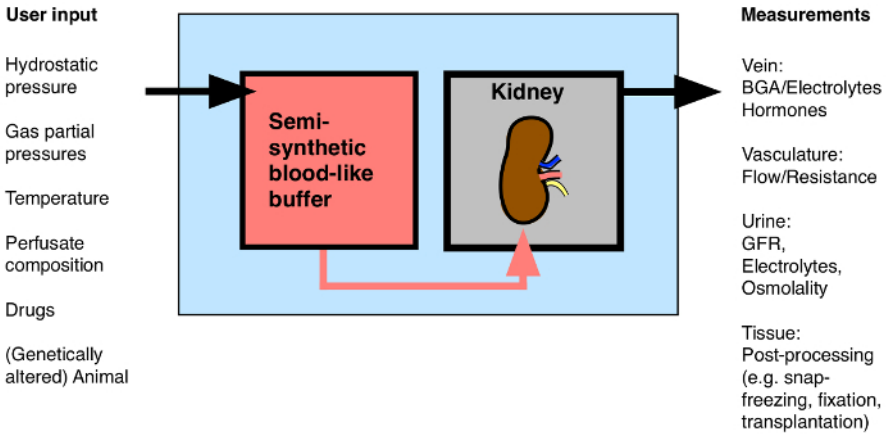

Figure 1: Overview of Possible Input/Output to the Isolated Perfused Kidney. *BGA*: Blood gas analysis. Please click here to view a larger version of this figure.

This technique likely will receive increasing attention over coming years, as many innovative applications are being discussed with the dawn of prolonged normothermic kidney perfusion prior to transplantation (with or without the application of anti-rejection or genome-editing drugs)[8,9,10,11], the bioengineering of whole kidneys from decellularized scaffolds[12], and the application of high doses of fluorescent dyes for multiphoton imaging[13]. It is also an ideal model with which to study the role of specific genes during acute kidney injury[14].

A step-by-step protocol is given to allow other laboratories to perform isolated mouse kidney perfusion successfully. First, the composition and preparation of the buffer is specified. Then, the surgery is described in detail and the critical steps are shown. Third, data is presented that are representative of a successful preparation: renal blood flow, vascular resistance, glomerular filtration rate , and fractional electrolyte excretion-all as functional measurements of viability-and transmission electron micrographs of the morphology of different nephron segments of perfused kidneys fixed after 1 hr of perfusion.

Protocol

All procedures involving animals described in this manuscript were conducted according to Swiss law and approved by the veterinary administration of the Canton of Zurich, Switzerland.

1. Buffer Preparation

1. Prepare solutions 1 - 4 and the antidiuretic hormone (ADH) solution (**Table 1**).
2. Prepare the dialysis buffer (**Table 1**).
   NOTE: This is the buffer used as the dialysis buffer during the perfusion. Later, the erythrocytes will be diluted in this buffer to form the final perfusate.
3. Erythrocyte preparation.
   1. Dilute 250 ml of human erythrocyte concentrate (tested material obtained from the local blood bank) to 500 ml with dialysis buffer. Centrifuge at 2,000 x g for 8 min. Remove the buffer, being careful not to remove any erythrocytes. Repeat 3x.
4. Prepare the albumin (bovine serum albumin; BSA) buffer.
   1. In 200 ml of dialysis buffer, dissolve 44 g of BSA using a stir bar. Filter the solution with filter paper.
5. Prepare the perfusate.
   1. Filter the erythrocytes from step 1.3.1 through filter paper into the BSA buffer. Fill up to a total volume of 800 ml with dialysis buffer.
      NOTE: This is the final perfusate. The hematocrit should now be between 8 and 12%. The perfusate can be stored for up to 12 hr at 4 °C.

2. Initiating Dialysis and Oxygenation

1. Turn on the water bath surrounding the larger buffer reservoir, smaller buffer reservoir, and the moist chamber (a small, double-walled chamber brought to 37 °C and 100% humidity to later hold the kidney) to 37 °C (**Figure 2**).
2. Fill the larger buffer reservoir with the dialysis buffer and the smaller reservoir with the perfusate.
3. Turn on the 5% CO_2/95% O_2 gas inflow to the dialysis buffer.
4. Switch on continuous dialysis of the perfusate against the dialysis buffer. Take care to use low-flux dialysis tubing. Proceed to Step 3.

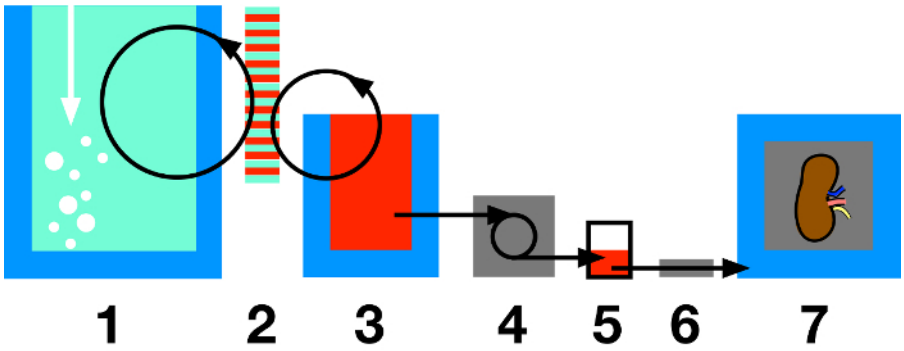

Figure 2: Schematic Drawing of the Perfusion Circuit. Scheme shows the main components of the perfusion circuit and the direction of buffer flow. All components surrounded by dark blue are kept at 37 °C with a water bath/thermostat. **1:** Dialysis buffer of at least 3 times the volume of the perfusion buffer is continuously bubbled with 95% O_2/5% CO_2. **2:** Dialysis buffer and perfusion buffer are continuously dialyzed against each other in a dialysis tube by a roller pump. **3:** Due to this dialysis, the perfusion buffer is enriched with 9% O_2/5% CO_2 and electrolyte levels are kept constant throughout perfusion. **4:** A roller pump propels the perfusion buffer toward the kidney. **5:** A windkessel removes peristaltic waves and acts as a bubble trap. **6:** Pressure transducer (connected to *4.* (roller pump) to keep continuous pressure while allowing freely alternating flow). **7:** Throughout perfusion, the kidney remains in a moist chamber for 100% air humidity and 37 °C kidney temperature. Please click here to view a larger version of this figure.

3. Surgical Procedure Part 1 (for a diagram of all ligatures, see Figure 3)

Note: Perform all ligatures using 5-0 surgical thread.

1. Anesthetize a mouse by intraperitoneal injection (10 µl/g of body weight, 20 mg/ml ketamine and 1 mg/ml xylazine dissolved in 0.9% NaCl). Confirm sufficient depth of anesthesia by testing for absence of rear-foot reflexes.
2. Fix the mouse in a supine position in the moist chamber. Protect the eyes with vet ointment. Place a 1 ml syringe below the spine to elevate the lumbar vessels.
3. Perform a median laparotomy from the pubic crest to the sternum opening first the skin, then the abdominal muscles, with scissors.
4. Remove the intestine and place it on the left side of the mouse lateral from the abdomen.
5. Free the bladder from connective tissue and explore both ureters and the urethra.
6. Place a ligature around the left ureter (ligature I). Close it.
7. Place a ligature around the urethra (ligature II). Close it.
8. Place a "lasso" ligature around the whole bladder (ligature III).
9. Incise the bladder 1 mm.
10. Cannulate the opening with 2 cm PE 50 tubing.
11. Close ligature III around the tubing.
12. Cut the left ureter and urethra distal from the ligatures. The bladder is now attached to the right ureter only and freely moving.
13. Clear the abdominal aorta of connective tissue and fat.
14. Place an abdominal mid-aorta ligature (ligature IV).
15. Place a ligature around the aorta below the diaphragm between the superior mesenteric artery and the coeliac trunk (ligature V).
16. Place a ligature around the superior mesenteric artery (ligature VI).
17. Place an aortic ligature directly below the right and above the left renal artery (ligature VII).
18. Place a ligature around the caudal vein package (cava) (ligature VIII). Proceed to Step 4.

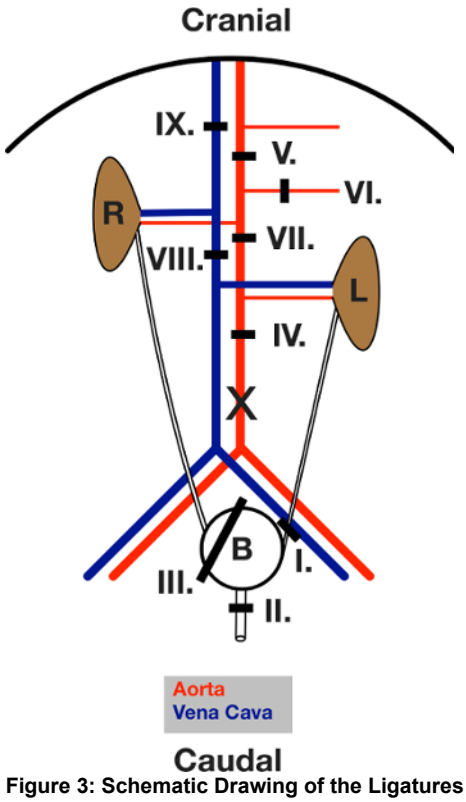

Figure 3: Schematic Drawing of the Ligatures placed during Surgery. View of the open abdomen after the laparotomy. The intestine is moved out to the left. *L* and *R* indicate the left and right kidney. The black lines show the area of the respective ligature. Ligatures are first placed and then closed, in the sequence given in the text. *X* marks the location of the incision for aorta cannulation. Please click here to view a larger version of this figure.

4. Priming of the Perfusion Circuit

1. Start the rotary pump and fill the tubing with perfusate. Take care to empty all air bubbles from it.
2. Fill the windkessel device to approximately mid-level with perfusate.
3. Calibrate the pressure transducer to 0 mm Hg when all tubing is filled and flow is 0. Keep the perfusion needle at kidney level during this time.
4. Keep flow at a constant minimal level (0.6 ml/min) and proceed to Step 5.

5. Surgical Procedure Part 2

1. Place a clamp between ligature IV and the branching of the left renal artery.
2. Make a small incision in the aorta caudal of ligature IV, taking care to not cut the dorsal wall.
3. Dilate the opening in the aorta with a vessel dilator.
4. Cannulate the aorta with a needle (2 cm long, pulled PE 50), pushing the tip just to the clamp.
5. Open the clamp.
6. Push the tip of the needle cranially until it reaches the junction of the right kidney artery and the aorta.
7. Close ligature VII.
8. Close ligature IV.
9. Open the chest with scissors by dissecting the diaphragm. With a single cut, separate the aorta, vena cava, heart and vegetative nerves. With this step, the animal is sacrificed via rapid exsanguination under continuous deep anesthesia.
10. Start pressure control of the perfusion pump. Maintain the mean pressure between 80 and 100 mmHg.
11. Close ligature V.
12. Close ligature VI.
13. Close ligature VIII.
14. Free the right kidney from connective tissue and its embedding into the adipose capsule with scissors.
15. Cut the aorta proximally to ligature V.
16. Cut the superior mesenteric artery distally to ligature VI.
17. Cut the kidney-supporting vessel bundle out, taking care to not cut into the vessels themselves.
18. Cut the liver at the connection to the kidney. Take care to free the kidney, but leave a small part of the liver adherent to it, so that the vena cava is kept open by it.
19. Take the kidney bundle out of the mouse. Remove the mouse from the moist chamber.
20. Place a "lasso" ligature around the connection of liver and kidney (ligature IX).
21. Cannulate the vena cava with a venous line (2 cm PE 50).
22. Close ligature IX. Venous outflow through the venous line should immediately start.

23. Close the moist chamber.

6. Downstream Analyses

1. During the following hour, continuously monitor blood flow and intravascular pressure[15]. Collect venous outflow, which can be used for analysis of, for example, renal renin release[7]. Collect urine for analysis of electrolyte concentration and glomerular filtration rate [14]. After 1 hr of perfusion, kidneys can be snap-frozen for western blotting or be fixed for imaging approaches[16].

Representative Results

With the method described, isolated mouse kidneys can remain viable for at least 1 hr. We tested the tissue viability after 1 hr of continuous perfusion with functional (renal blood flow and vascular resistance, blood gas analysis of venous outflow, glomerular filtration rate, urinary fractional Na^+ and K^+ excretion, and urine osmolality) and morphological (transmission electron microscopy, TEM) methods in four kidneys of wildtype C57Bl/6 mice. Additionally, western blots of apical marker proteins of different tubule segments were performed, comparing isolated perfused kidneys to unperfused kidneys of the same animals. In all experiments, constant pressure perfusion was used, keeping the perfusion pressure at ~ 100 mmHg (**Figure 4**). In those kidneys, perfusate flow and vascular resistance remained stable over a time of 55 min at 15.6 ± 0.4 ml/min*g of kidney weight and 35 ± 1.0 mmHg*min/ml, respectively (**Figure 4 A**). A decrease of perfusate flow or an increase in vascular resistance is a sensitive parameter with regards to decreasing tissue viability and should be monitored throughout the perfusion with care. Blood gas and electrolyte analysis in 100 µl samples taken 60 min after the onset of perfusion revealed renal CO_2 production and O_2 consumption, when the arterial inflow was compared to the venous outflow (**Table 2**). The other parameters remained unchanged between artery and vein (**Table 2**). Special emphasis should be placed on unaltered venous potassium release, as an increase may occur during tissue damage. Glomerular filtration rate, assessed via FITC-Inulin clearance[14], tended to increase over time (**Figure 4 B**) but this trend did not reach significance. Fractional excretion of sodium and potassium assessed after 55 minutes of perfusion is increased compared to the *in vivo* situation (**Figure 4 C**). Urine osmolality is not elevated above the osmolality of the venous outflow after 55 min of perfusion (**Figure 4 D**).

For morphological studies, kidneys were fixed after perfusion in 3% paraformaldehyde and 0.1% glutaraldehyde in dialysis buffer as previously described[17]. Renal ultrastructure was assessed with TEM. Glomeruli and S1 segments of proximal tubules appeared largely unaltered after perfusion (**Figures 5 A and B**). Some, but not all S2/S3 segments of proximal tubules and the thick ascending limbs that were not close to vascular bundles were showing signs of organ damage, as described previously in rat isolated perfused kidneys[5] (**Figures 5 C and D**). Even upon close inspection, distal convoluted tubules and collecting ducts did not show evident signs of necrosis (**Figures 5 E, F, and G**). In conclusion, the morphological findings in mouse kidneys mirror the situation found in the rat isolated perfused kidney[5].

Western blots of apical marker proteins of the different nephron segments in isolated perfused kidneys (snap-frozen after 1 hr of perfusion) compared to unperfused contralateral kidneys of the same animal (snap-frozen before the onset of perfusion) did not reveal significant alterations. We studied NaPi-IIa (located in proximal tubules), NKCC2 (thick ascending limbs), NCC (distal convoluted tubules), and ENaC (connecting tubules/collecting ducts) **(Figures 6 A and B**).

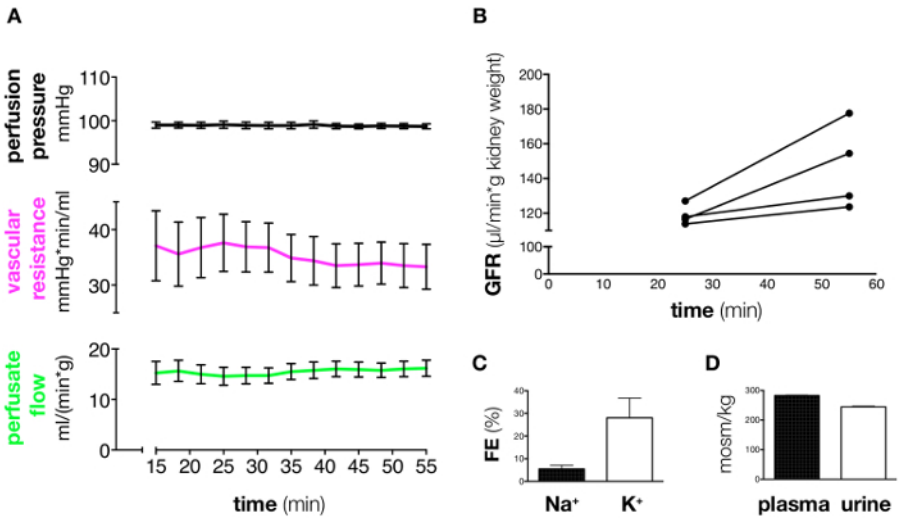

Figure 4: Assessment of Functional Viability of Kidneys Perfused for 55 min. A) During constant pressure perfusion, vascular resistance and perfusate flow remained stable for 55 min. The first 15 min are excluded due to the high variability always seen during this time. **B)** Glomerular filtration rate (FITC-Inulin) of four kidneys assessed after 25 and 55 min displayed slight but not significant increase over time. **C)** Fractional excretion of sodium and potassium assessed after 55 min of perfusion. **D)** Venous plasma and urine osmolality after 55 min of perfusion. n = 4 in all experiments. Data are presented as means ± SEM. Please click here to view a larger version of this figure.

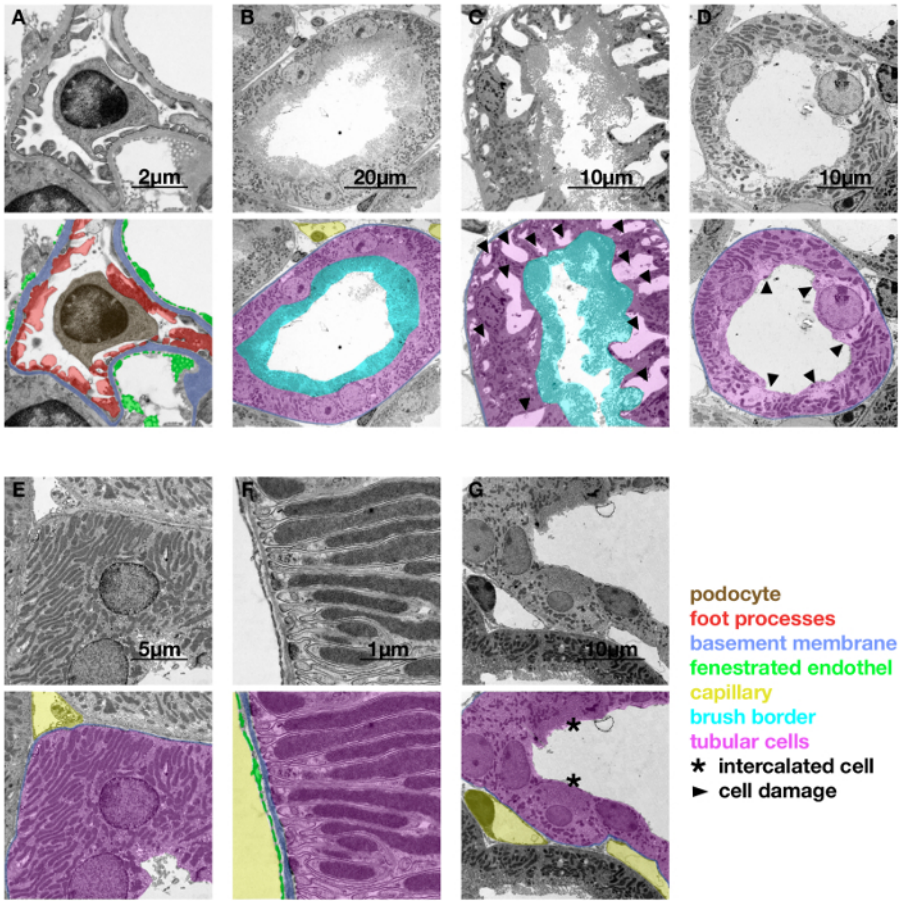

Figure 5: Transmission Electron Microscopy. Representative Morphology of Different Nephron Segments of Perfused Kidneys at an End Point of 1 hr. A) Glomeruli are intact. Detail of a podocyte in a glomerulus. **B)** S1 segment of a proximal tubule. S1 segments are intact. **C)** S2/S3 segment of a proximal tubule. Some, but not all S2/S3 segments of proximal tubules are highly necrotic. Cells appear swollen and show large intracellular damage. The tubular outline is maintained only by the basement membrane. **D)** Thick ascending limb (TAL). Those TALs that are far from the vascular bundles show the first signs of hydropic degeneration. Luminal membranes appear thinned and fragile. **E)** Distal convoluted tubule (DCT). DCTs are intact. **F)** Detail of the basolateral infoldings of a DCT cell. Fenestrated endothelium, basement membrane, basolateral infoldings, and mitochondria are intact. **G)** Collecting duct (CD). CDs are intact. Please click here to view a larger version of this figure.

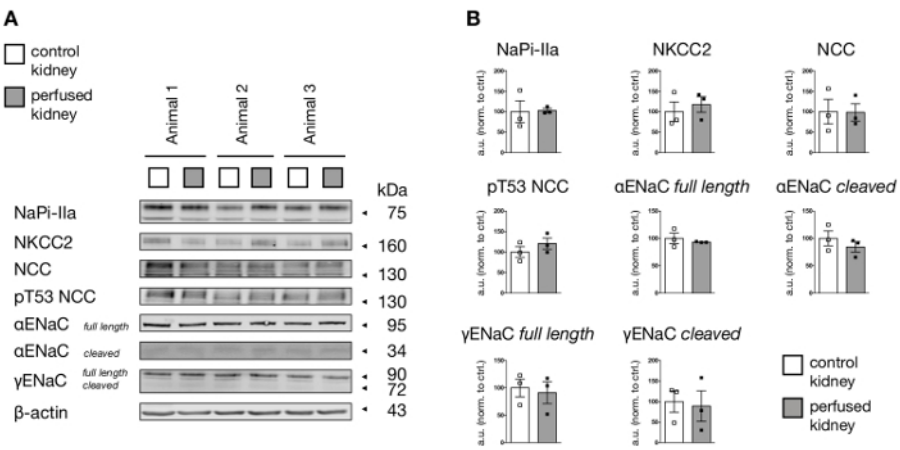

Figure 6: Western Blot of Apical Marker Proteins in Isolated Perfused Kidneys Compared to Unperfused Kidneys from the Same Animals. A) Western blots of kidneys perfused for 1 hr and unperfused kidneys. NaPi-IIa is representative for the proximal tubule. NKCC2 is representative for the thick ascending limb. NCC and NCC phosphorylated at threonine 53 (pT53 NCC) are exclusively found in the distal convoluted tubule. Alpha and gamma subunits of ENaC in their full length and their respective proteolytically cleaved products are shown representative for the connecting tubule/collecting duct. **B)** Quantification of A) did not reveal significant differences between kidneys perfused for 1 hr and unperfused kidneys. n = 3 in all experiments. Data are presented as means ± SEM and individual values are shown. Please click here to view a larger version of this figure.

Solutions:	Chemical/Drug	Mass/Volume	Concentration in final dialysis buffer (mM)
Solution 1 (10x concentrated)	NaCl	126g	107.8
	$NaHCO_3$	46g	27.4
	KCl	6.6g	4.42
	Urea	7.2g	6
	Creatinine	0.5g	0.132
	Ampicillin	0.3g	
	$MgCl_2 * 6H_2O$	4g	1
	add dH_2O to a final volume of 2l		
Solution 2 (10x concentrated)	Glucose	15g	8.32
	add dH_2O to a final volume of 1l		
Solution 3 (10x concentrated)	$CaCl_2 * 6H_2O$	2.6g	0.935
	add dH_2O to a final volume of 1l		
Solution 4 (10x concentrated)	NaH_2PO_4	0.4g	2.88
	Na_2HPO_4	1.2g	0.66
	add dH_2O to a final volume of 1l		
Antidiuretic hormone (ADH) solution	ADH	1µg	
Dialysis buffer	Solution 1	500ml	
	Solution 2	500ml	
	Solution 3	500ml	
	Solution 4	500ml	
	Aminoplasmal	83ml	
	ADH solution	0.5ml	
	Na-Pyruvate	165mg	0.3
	Na-Glutamate	262mg	0.31
	Na-Malate	281mg	1.15
	alpha-Ketoglutarate	1133mg	1.2
	Na-Lactate	1176mg	2.1
	FITC-Inulin	25mg	
	Add dH_2O to a total volume of 5l		
	Adjust pH to 7.7		
Perfusion buffer (for 1 mouse kidney)	Dialysis buffer	400ml	
	Human erythrocytes	to hct of 10%	

Table 1: Composition of Solutions and Dialysis Buffer. Please click here to view a larger version of this table.

Parameter	Artery	Vein
pH	7.38 ± 0.03	7.38 ± 0.01
pCO_2 (kPa)	3.73 ± 0.21	4.28 ± 0.25
pO_2 (kPa)	55.16 ± 1.5	6.26 ± 1.0
Na^+ (mmol/l)	152.8 ± 2.0	153 ± 1.9
K^+ (mmol/l)	4.2 ± 0.1	4.2 ± 0.1
Ca^{2+} (mmol/l)	1.1 ± 0.2	1.1 ± 0.2
Cl^- (mmol/l)	107 ± 1.7	106.6 ± 1.5
HCO_3^- (mmol/l)	21.9 ± 0.5	22.1 ± 0.6

Table 2: Blood Gas and Electrolyte Analysis of Arterial Inflow and Venous Outflow of Perfused Mouse Kidneys 60 min after the Onset of Perfusion (n= 4). Data are presented as means ± SEM.

Discussion

The mouse isolated perfused kidney is a tool for studying kidney function in a controlled environment *ex vivo* for 1 hr, bridging the gap between *in vivo* experiments in intact animals, which may be flawed by the impact of numerous systemic factors, and *in vitro* experiments in isolated nephron segments or cultured cells, which necessarily neglect the impact of intact organ structure on function. There is, to the authors' knowledge, no alternative technique with which to perform this specific task. Biochemical studies on kidney tissue, however, can be performed in a much simpler fashion using kidney slices[17]. While the technique presented is currently used mostly to study renal physiology, many new applications are being discussed. For example, it could be used for kidney bioengineering during perfusion decellularization and recellularization[12]. In addition, prolonged normothermic perfusion of kidneys prior to transplantation, with the administration of high doses of anti-rejection or genome-editing drugs into the perfusate, is being studied; even in clinical settings[8, 9, 10, 11]. The mouse isolated perfused kidney is an excellent tool that could be used for these applications, especially to elucidate the role of individual genes with knockout models.

Three steps are critical for a successful experiment. This paper provides interested labs with the means to reproduce the procedure. First, the buffer needs to be prepared in such a way that the kidney receives most of the nutrients necessary for prolonged viability under reproducible

conditions. A balance must be found between fully controlled conditions (synthetic buffer) and a native perfusion condition (whole blood). Therefore, albumin and mammalian erythrocytes to a hematocrit of 10% have to be included. This hematocrit represents, in the authors' experience, the best compromise between sufficient tissue oxygenation and low buffer viscosity (increasing hematocrit decreases flow dramatically during pressure-constant perfusion (compare values presented in this paper to values in kidneys perfused without erythrocytes[18])). The quality and freshness of the erythrocytes is critical. During the washing steps, erythrocytes need to be handled with care to prevent hemolysis. The buffer needs to be prepared freshly on the day of the experiment. Second, the surgery needs to be carried out by a trained surgeon within the shortest time possible. The surgeon needs to perform 6 ligatures around the main vessels connecting the kidney to the rest of the body, while keeping the animal's blood loss to a minimum. For optimal results, the surgical interventions should be accomplished within 30 minutes. If the mean arterial blood pressure of the animal drops, during prolonged surgery or due to anesthesia, below 60 mmHg, renal perfusion will already be minimal and the tissue will be prone to necrosis. To rule this out, the left kidney can be taken after surgery, when perfusion of the right kidney has already begun, and used as an untreated control. Third, during the time of the perfusion the correct functioning of the perfusion apparatus, consisting of a standard small-animal-perfusion circuit, needs to be ensured. While the computer performs the perfusion by itself, human supervision is necessary, for example to check for bubbles in the perfusion circuit.

The representative data provided allows for the assessment of kidney viability after 1 hr of *ex vivo* perfusion and the comparison of the technical success of any execution of this method to the approach described herein. During at least 1 hr, renal blood flow, vascular resistance, blood gases of the venous outflow and glomerular filtration rate should remain stable.

There are certain limitations to the technique, which one has to keep in mind. Even a kidney issuing from the most successful surgery can only remain viable for a limited time. The authors found 1 hr to be the best time point at which to stop perfusion. Additionally, while perfusion of the isolated kidney allows for the study of the isolated whole organ in depth, it is difficult to differentiate specific effects on one cell type. Kidneys fixed after 1 hr might show defects in S3 segments of proximal tubules and thick ascending limb cells, while glomeruli, S1 segments, DCTs, and CDs should remain intact. The obligatory use of erythrocytes, especially from humans, is also linked to potential risks (*e.g.*, infections) for the experimenter.

In summary, the mouse isolated perfused kidney is a useful technique with which to study the whole organ *ex vivo*. Like any technique, it has certain advantages and limitations. The authors believe that both the manifold new applications currently arising and this protocol could provide sufficient momentum for other labs to reproduce this effort.

Disclosures

The authors have no competing financial interests and nothing else to disclose.

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