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ORIGINAL ARTICLE

Biopsychronology: live confocal imaging of biopsies to assess organ function

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Summary

Prolonged ischemia (I) times caused by organ procurement and transport are main contributors to a decrease in organ function, which is further enhanced during reperfusion (R). This combined damage, referred to as ischemia–reperfusion injury (IRI), is a main contributor to delayed graft function, which leads to costly and lengthy follow-up treatments or even organ loss. Methods to monitor the status of a graft prior to transplantation are therefore highly desirable to optimize the clinical outcome. Here, we propose the use of fine needle biopsies, which are analyzed by real-time live confocal microscopy. Such a combination provides information about the functional and structural integrity of an organ within a few minutes. To confirm the feasibility of this approach, we obtained fine needle biopsies from rodent kidneys and exposed them to various stress conditions. Following the addition of a range of live stains, biopsies were monitored for mitochondrial function, cell viability, and tissue integrity using confocal live cell imaging. Our data demonstrate that this procedure requires minimal time for sample preparation and data acquisition and is well suitable to record organ damage resulting from unphysiological stress.

Introduction

During solid organ transplantation, ischemia (I) and subsequent reperfusion (R) trigger a series of events that culminate in severe injury to the graft (ischemia–reperfusion injury – IRI) [1]. Various players have been implicated in the establishment of this damage, which is caused initially by the collapse of mitochondrial homeostasis and enhanced at later time points by inflammation. While ischemia is the trigger for IRI, reperfusion, although essential for prolonged graft survival, is seen as the main cause [1,2]. Mitochondrial reactive oxygen species (ROS) production occurs immediately after reperfusion resulting in cellular damage and perturbation of Ca^{2+} homeostasis (mitochondrial Ca^{2+} overload) leading to apoptotic or necrotic cell death [1–6]. One eminent challenge is to predict a decline in donor organ function prior to transplantation, which in addition to extended ischemia times also may be affected by donor age, medical history, and other variables. Due to organ shortage, donor criteria have been extended and borderline quality organs are included, thereby increasing the need for the assessment of organ function prior to transplantation. In the clinical routine, organ function after transplantation is mainly assessed by monitoring functional or damage parameters and in selected cases by histological analysis. In the case of the kidney, estimated glomerular filtration rate

(eGFR) based on serum creatinine level is the most commonly used end point of renal graft function [7–10]. Histopathological analyses of the pretransplant donor renal biopsies (PTDB) are often performed in case of marginal donors [11,12]. However, as their analysis is time-consuming, they result in delayed transplantation and thus further deterioration of the graft. Therefore, there is a demand for methods that yield fast information about the integrity and quality of the donor organ. Here, we propose a minimally invasive approach, which may achieve just that by monitoring the preservation of key cellular functions such as mitochondrial membrane potential as well as viability and structural integrity. Our approach is based on the acquisition of biopsies, incubation with appropriate reporter dyes, which monitor the functional status of viable cells, followed by real-time live confocal analysis.

Events occurring during ischemia and early reperfusion, in particular, affect mitochondrial function. IR is linked to inadequate ATP production through mitochondrial oxidative phosphorylation leading to a collapse of the mitochondrial membrane potential [13,14]. IR-induced ROS formation contributes to acute apoptotic or necrotic cell death [15]. In addition to ROS, nitric oxides (NO) are robustly produced during the early phase of reperfusion. The impact of NO, protective or cytotoxic, depends on the site and rate of NO production and concomitant presence of ROS [16]. The combination of live cell staining, using fluorescent probes which respond to changes in mitochondrial membrane potential, ROS and NO, with live confocal microscopy allows rapid nondestructive serial recording of cell viability parameters in real time [17,18]. Renal function is crucially dependent on renal structure, which provides the basis for the regulatory mechanism that controls the transport of water and solutes between filtrate and plasma and the urinary concentration [19]. Therefore, we used FITC-labeled wheat germ agglutinin (WGA), which allows for monitoring of cell and tissue morphology [17,20]. To discriminate between living and dead cells, we added the nuclear stains Syto 16, staining all nuclei, and propidium iodide (PI) only staining those of dead cells [21].

To obtain proof that highly intact biopsies can be obtained which allow recording of stress responses, murine or rat kidneys were either left untreated or exposed to ischemia of 35 min followed by 12 min of reperfusion. After these steps, fine needle biopsies were taken and analyzed. Our findings demonstrate that this approach, which we call "biopsychronology," requires minimal time for sample preparation and is well suitable to record damage resulting from unphysiological stress to the organ. We advertise its use for the *ex vivo* assessment of organ function in humans prior to transplantation, but also in the setting of experimental transplantations in animal models. As biopsies also can be cultured *in vitro,* this approach may be helpful in the *in vitro* pretesting of therapeutic interventions.

Material and methods

Animal experiments

Male Lewis rats (250–300 g) and C57Bl/6N mice (23–27 g) were obtained from Charles River, Germany, and kept with unlimited access to water and standard laboratory chow. All experiments were approved by the Austrian Ministry of Education, Science and Culture and were performed in accordance with national animal protection guidelines as well as the "Principles of Laboratory animal care" (NIH publication Vol. 25, No.28 revised 1996). Prior to the induction of IR, mice were anesthetized with isoflurane and placed on a heating pad to maintain their core body temperature at 37 °C during surgery. A middle incision was made to expose the abdominal cavity, and left renal pedicle was freed of the surrounding tissue by blunt dissection. A nontraumatic vascular clamp was placed on the pedicle for 35 min to induce ischemia in the left kidney, and for subsequent reperfusion, the clamp was removed. Renal occlusion was macroscopically verified by the change in color of the kidney to pale and reperfusion by a blush appearance. After 12 min of reperfusion, the left kidney was harvested and used to take needle biopsies for live confocal imaging. The right kidney served as a control. For each experiment, 4 technical replicates of mice or rats were performed.

Sampling and handling of renal biopsies

The renal biopsies were performed either with 18-G biopsy needles (Super-Core Semi-automatic Biopsy Instrument, Angiotech, PBN Medicals, Stenløse, Denmark) or with scalpels to obtain longitudinal biopsy cylinders. After carefully removing the renal capsular layer, four biopsies were independently taken from each kidney. To avoid any mechanical stress/damage, biopsies were carefully picked by forceps at the hilus end of the biopsy and transferred to the eight-well chambered cover glass (Nalge Nunc International, Naperville, IL, USA).

Biopsies taken from rat kidneys were either directly stained with different fluorescent probes as described below or incubated in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich, St. Louis, MO, USA) containing 15 mM HEPES, 15 mM pyridoxine, and $NaHCO_3$, supplemented with 5% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria), penicillin (100 U/ml) (PAA Laboratories), streptomycin (100 μg/ml) (PAA Laboratories), and 2 mM L-glutamine (GIBCO Invitrogen, Grand Island, NY, USA) for 24 h in at 37 °C, 5% CO_2, 95% air, followed by loading with appropriate fluorescent probes and live confocal

microscopy. To completely permeabilize the cell membranes, needle biopsies were incubated with digitonin (final concentration 10 μg/ml) for 5 min. Prior to confocal analysis, the medium containing the fluorescent dyes was exchanged with fresh medium without dyes. Five independent random areas were analyzed for each biopsy.

Assessment of cellular function and integrity using live stains

By combining membrane-permeable monovalent cationic fluorophores with spinning disk confocal microscopy, mitochondrial function can be analyzed in cells as well as tissue samples [17,18]. Viable mitochondria were stained with tetramethylrhodamine methyl ester (TMRM, Sigma-Aldrich; 50 nM final concentration), the plasma membrane with wheat germ agglutinin conjugate (WGA, Molecular Probes, Eugene, OR, USA; 10 μg/ml final concentration), all nuclei with Syto16 (Molecular Probes; final concentration 5 μM), and the nuclei of the dead cells with propidium iodide (Molecular Probes; final concentration 500 nM) [21]. For all stains, the incubation time was 15 min in a cell culture incubator (37 °C, 5% CO_2). Intracellular ROS and NO were measured as described previously [22]. Briefly, kidney biopsies were loaded with 10 μM DCF-H_2-DA (Molecular Probes Inc.) or 10 μM DAF-2-DA (Calbiochem, San Diego, CA, USA), dissolved in aforementioned DMEM/F12 culture medium, for 15 min at 37 °C, 5% CO_2 to measure ROS and NO, respectively. In most cases, biopsies were also co-loaded with 50 nM TMRM to simultaneously measure mitochondrial transmembrane potential along with ROS or NO.

Real-time live confocal imaging

Real-time live confocal imaging was performed in eight-well chambered cover glass (Nalge Nunc International). After the incubation time with the live stains, the medium was changed and the tissue biopsies were immediately imaged. Confocal images were acquired with a microlens-enhanced Nipkow disk-based UltraVIEW RS confocal scanner (Perkin Elmer, Wellesley, MA, USA) mounted on an Olympus IX-70 inverse microscope (Olympus, Nagano, Japan). Images were collected using the ULTRAVIEW LCI software version 5.4 (Perkin Elmer).

Results

We used rat kidney biopsies to develop methods for analyzing their functional status and integrity, an approach which then should be easily transferable to the human setting. This procedure results in good maintenance of organ substructures (e.g., glomerular and tubular structures), and biopsies can be taken in a way that a cross section of the kidney can be obtained (Fig. 1), thereby maximizing the information that is obtained in a single analysis. Care was taken to minimize handling time, and all biopsies were analyzed within 30 min.

As the dyes used monitor parameters, which are affected by stress, it is important to avoid any additional damage during and after harvesting the biopsy. To keep the pH stable, biopsies were stained in a HEPES-buffered culture medium.

In our study, we used five different stains: (i) tetramethylrhodamine methyl ester (TMRM), a dye that is readily sequestered by functioning mitochondria with intact mitochondrial membrane potential [18], (ii) propidium iodide (PI), which is only taken up by nuclei of dead cells, (iii) Syto16, which stains nuclei in dead and living cells [21], (iv) DAF-DA, which becomes fluorescent in the presence of nitric oxide (NO) [23], and (v) FITC coupled wheat germ agglutinin conjugate (WGA), which binds to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid residues, thereby staining cell membranes as well as matrix components [17,18,20] and thus can be used to monitor the intactness of the tissue. Moreover, using appropriate fluorophores, these dyes may be combined for simultaneous detection of various parameters. A flow scheme of our approach is depicted in Fig. 1. Following rat kidney biopsy, samples were incubated with the live stains Syto 16, PI, and WGA and immediately processed for imaging. Incubation time was generally 15 min although shorter incubation times can also be used as the fluorescence is already present after a few minutes (data not shown). Serial images were taken along the whole length of the biopsy allowing for the coverage of a cross section through a large area of the organ (Fig. 1). An insert depicting tubuli in the cortical area of the kidney is shown in the upper left corner of the Fig. 1, and one showing a glomerulus is inserted in the lower right corner.

In a first set of experiments, we either analyzed biopsies immediately after biopsy (i.a.b.) or performed *in vitro* manipulations to confirm that this approach is suitable to monitor changes in the cellular environment. For this purpose, rat kidney biopsies were analyzed after a 24-h incubation step at 37 °C in the tissue culture incubator (24 h 37 °C) or following permeabilization of the cell membrane by digitonin treatment, which results in the collapse of cellular homeostasis and cell death [24]. The pictures shown in Fig. 2 focus on the corticular area of the kidney and comprise several tubuli. Double staining with either Syto 16 (green) or PI (red; Fig. 2a–c) or TMRM (red) and WGA (green; Fig. 2d–f) was performed. Despite immediate processing of the samples (i.a.b), we observe heterogeneity in the staining pattern resulting from the presence of viable and nonviable regions in the tubular area. After the 24-h incubation step at 37 °C, the number of PI-positive (dead)

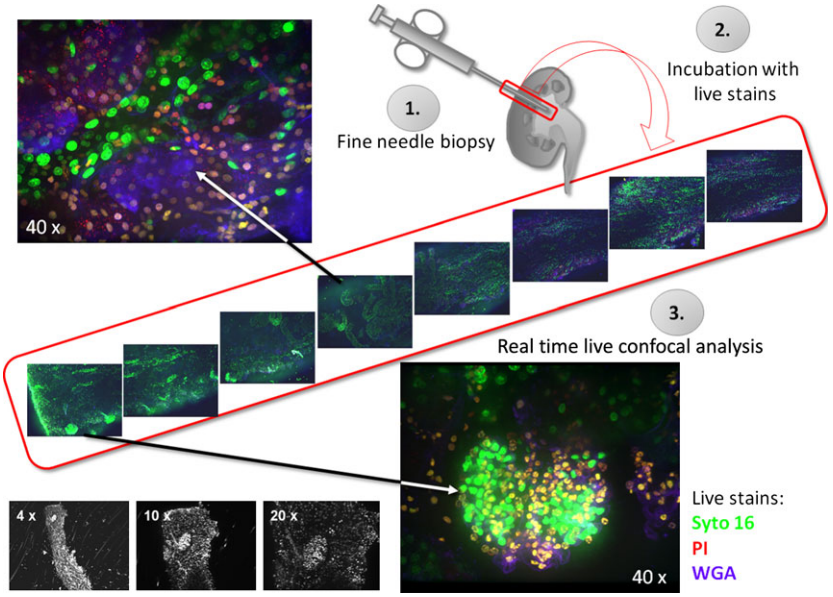

Figure 1 The three steps to "biopsychronology." In a first step, a fine needle biopsy is taken (shown is an example of a rat kidney). The second step consists of an incubation of the whole kidney biopsy with live stains. Stains can vary, depending on the cell viability parameters which are to be analyzed. In this case, the biopsy was stained with Syto16 (in green, staining all the nuclei), propidium iodide (PI, in red, staining the nuclei of the nonvital cells), and wheat germ agglutinin (WGA, in violet, staining the tissue morphology of the kidney sample). In the third step, real-time live confocal analysis with a spinning disk system is performed. Due to the spinning disk technology, the confocal analysis can be performed with a nonfixed biopsy sample. As the whole kidney biopsy is present, its orientation can be achieved by aligning serial pictures using low magnifications. Starting with low magnifications (see bottom left corner showing images acquired with a 4×, 10× and 20× objective), a more detailed analysis of regions of interest can be achieved using higher magnifications as exemplified with a 40× water immersion objective (see two examples at the top left and right bottom corner of the figure).

cells increases substantially, while a fraction of Syto 16-positive/PI-negative cells is still present at the end of the incubation period. After the digitonin addition, all nuclei are PI/Syto 16 positive resulting in a yellow overlay color. A similar picture emerges when looking at the mitochondrial membrane potential. At the earliest time point after the biopsy already, areas with decreased mitochondrial function are detectable (Fig. 2d), while TMRM staining intensity has decreased in almost all areas after the 24-h incubation step at 37 °C (Fig. 2e). Finally, the mitochondrial transmembrane potential has completely collapsed after the permeabilization of the cell membrane by digitonin (Fig. 2f).

The staining pattern obtained with WGA shows the same heterogeneity as does the staining with TMRM. Already immediately after biopsy (Fig. 2a), the tubules are differentially stained, depending on the type of tubule. In spite of this heterogeneity, the tissue integrity is clearly visible. After cultivation for 24 h at 37 °C, the structural integrity of the biopsy was lost and increased cell death was apparent as documented by Syto16/PI staining. Permeabilization with digitonin finally resulted in massive cell death (Fig. 2c) but had no influence on the staining pattern of WGA (Fig. 2f).

We next were interested to see whether our method is suitable to monitor cellular responses to cellular stress as it occurs in the clinical setting. For this purpose, we used murine kidney clamping as described in Material and Methods to cause ischemia in the organ. This was followed by a brief period of reperfusion, where usually damage initiated during ischemia becomes manifested. Following this, fine needle biopsies were taken and incubated with Syto 16 (green), propidium iodide (PI; red), and wheat germ agglutinin (WGA, blue). Real-time live confocal images of the corticular region depicting a glomerulus are shown in Fig. 3. The comparison of PI and Syto16 stain reveals substantial cell death in this sample, most likely resulting from the IR protocol applied.

To demonstrate the suitability of our method to monitor the status of a graft prior to transplantation, murine kidney was exposed to 0/6/24 h of cold ischemia. Following this, longitudinal biopsies were taken and incubated with Syto 16 (green) and propidium iodide (PI; red). With increasing time of cold ischemia, the amount of PI-positive nuclei increases indicating a rise in the number of nonviable cells (Fig. 4).

In a final set of experiments, we turned to a more detailed analysis of parameters, which should change as a result of increased stress to an organ. Again, we used the kidney clamping model in the mouse and dyes suitable for the

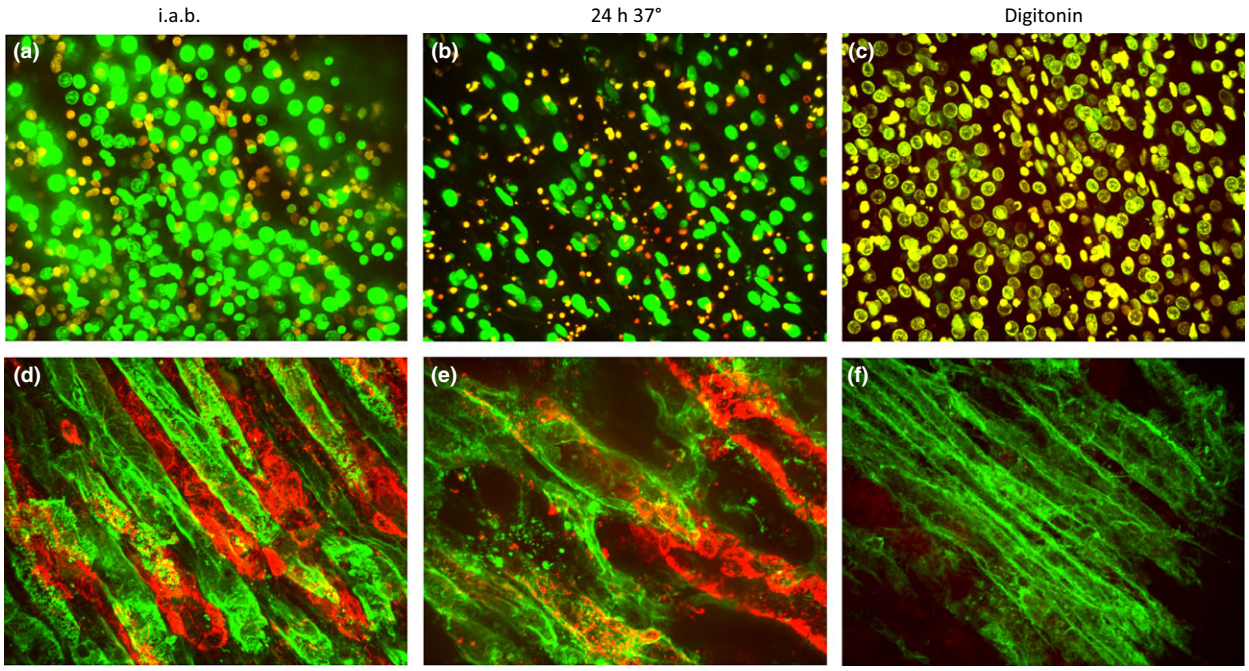

Figure 2 Real-time live confocal imaging of the corticular area showing several tubuli. Rat kidney biopsies were analyzed under three different conditions: Immediately after the biopsy (i.a.b.); 24 h incubation at 37 °C (24 h 37 °C) in tissue culture medium or after digitonin treatment (digitonin). (a–c) double staining with the two live stains Syto 16 (green) and propidium iodide (PI; red). (d–f) TMRM staining (red), WGA (green). All images were acquired with a 40× water immersion objective and consist of z-stacks of 25 planes with a spacing of 1 μm.

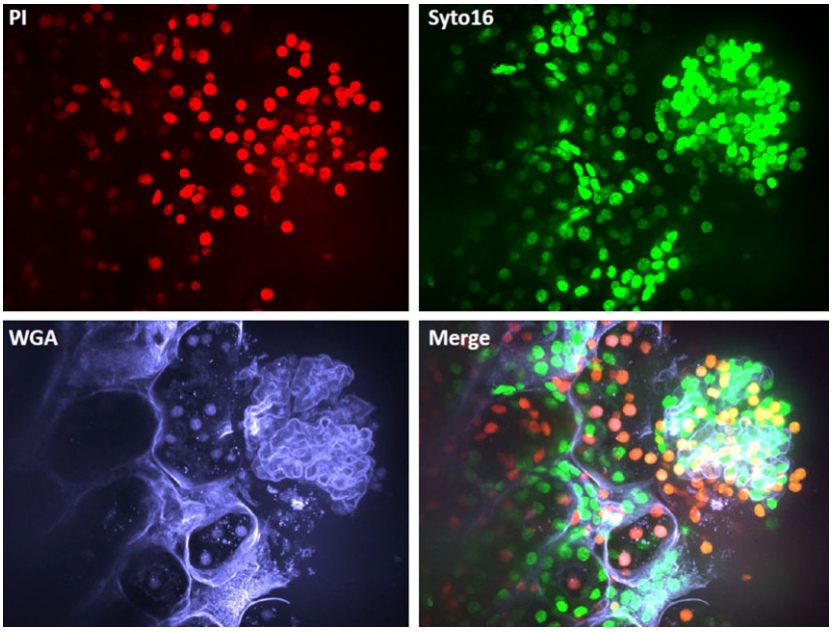

Figure 3 Real-time live confocal imaging of the corticular region showing a glomerulus. Following left renal IR (35 min), fine needle biopsies of murine kidneys were taken and incubated with the live stains Syto 16 (green), propidium iodide (PI; red), and wheat germ agglutinin (WGA, blue), thereby staining all the nuclei, the nuclei of the dead cells, and the tissue morphology. Images were acquired with a 40× water immersion objective. One representative example is shown consisting of a z-stack of 25 planes with a spacing of 1 μm.

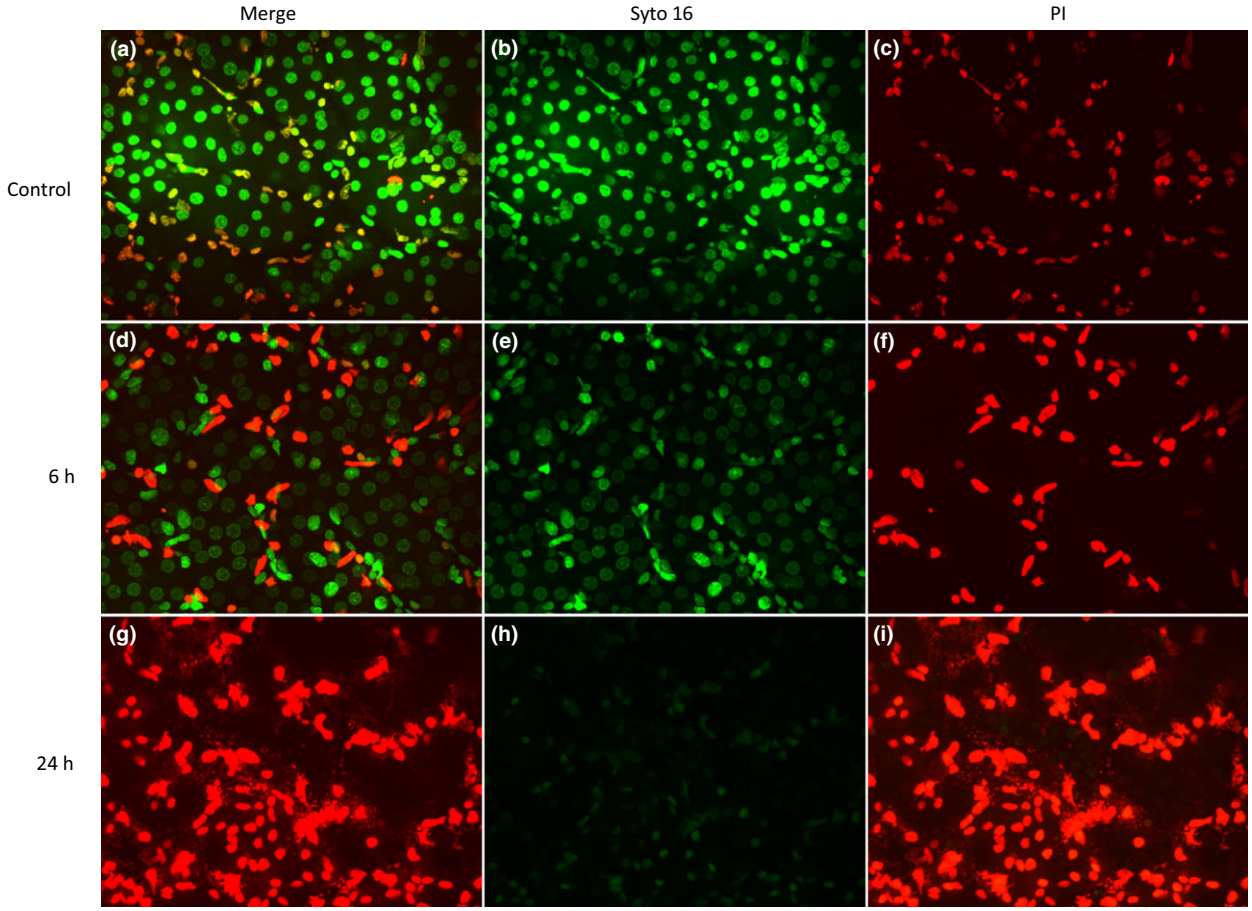

Figure 4 Real-time live confocal imaging of a longitudinal biopsy cylinders. Following 0 h (a–c), 6 h (d–f), and 24 h (g–i) of cold ischemia, longitudinal murine kidney biopsies were taken and incubated with the live stains Syto 16 (green), propidium iodide (PI; red). Images were acquired with a 40× water immersion objective. Representative examples are shown consisting of z-stacks of 25 planes with a spacing of 1 μm.

detection of ROS, NO, and mitochondrial transmembrane potential. Fine needle biopsies were taken from both, the left (IR) and right (control) kidney, and stained with DCF, DAF, or TMRM for the determination of intracellular ROS (Fig. 5a and b), NO (Fig. 5c and d), and mitochondrial transmembrane potential (Fig. 5e and f), respectively. Representative confocal micrographs depicting the fluorescence intensities of the control (left panels) and IR (right panels) kidney biopsies are shown in Fig. 5. Both ROS and NO levels increase as a result of IR, while the mitochondrial membrane potential was high in the control kidney and then collapse upon IR.

Discussion

Assessing organ function prior to transplantation holds the promise that the likelihood of subsequent graft failure with a requirement for additional organs or costly follow-up treatments is reduced. The method proposed by us combines different markers, which monitor the functional and structural status of an organ to be transplanted and should also be applicable to every organ or tissue. The use of biopsies combined with live cell staining and confocal imaging minimizes the amount of tissue and time required, while maximizing the area surveyed and the information gained. Applying this method, for which we propose the term 'biopsychronology', cell viability, tissue integrity, and mitochondrial status within different areas of biopsy can be imaged in a precise manner. In addition, staining as well as analysis can be performed in <20 min. Quantification of cell viability can be easily performed by comparing the numbers of Syto 16- and PI-positive nuclei. Moreover, the good preservation of the structure yields additional information about the status of the organ or specific structures within. Apart from the proposed clinical use in the setting of organ transplantation, this method is also highly suitable for animal studies. Moreover, the option to culture biopsies opens up the possibility to perform *ex vivo* experiments to gain detailed insights into processes which shape up organ damage

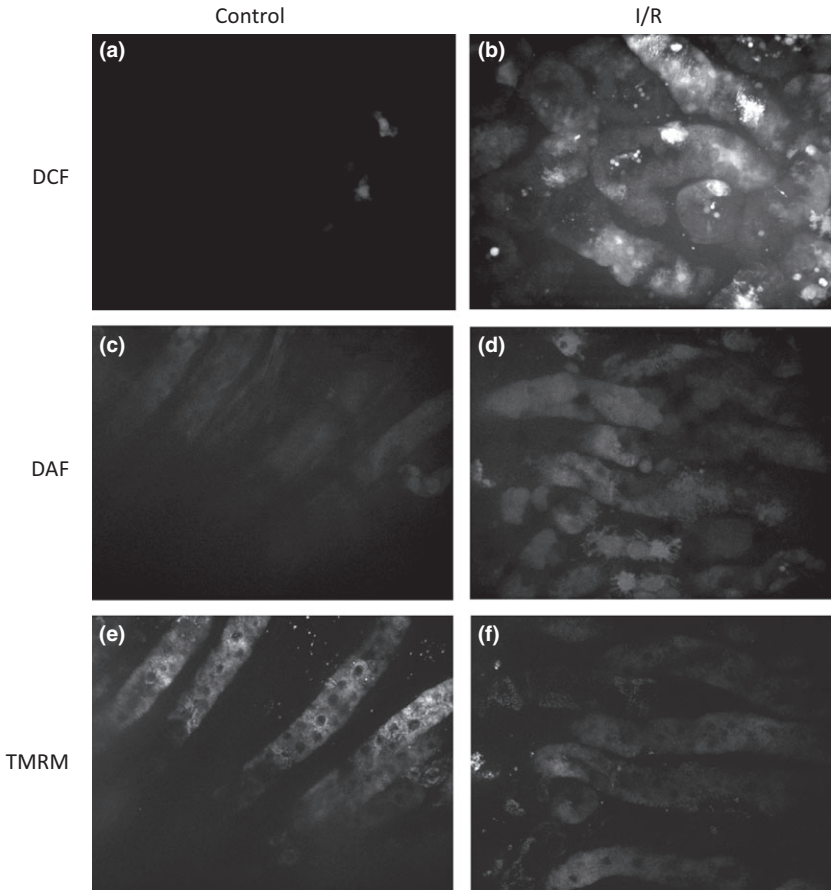

Figure 5 Detection of ROS, NO and mitochondrial transmembrane potential in murine kidney biopsies following IR. Following left renal IR in the mice as described in the Material and Methods, fine needle biopsies were taken from both the left (IR) and right (control) kidneys and stained with DCF, DAF, and TMRE for the determination of intracellular ROS (a, b), NO (c, d), and mitochondrial transmembrane potential (e, f), respectively. Representative confocal micrographs depicting the fluorescence intensities of the control (left panel) and IR (right panel) kidney biopsies are shown. Images show z-stacks of 25 planes with a spacing of 1 μm and were acquired with a 40× water immersion objective.

under cellular stress (e.g., hypoxic or toxic conditions) or to do a first testing of intervention strategies.

One striking observation is the heterogeneity, which is present in the biopsies from naïve organs but is also seen in the response to stress stimuli. Heterogeneity denotes the observation that cells or regions within an organ differ in staining intensity. Apart from the trivial possibility that this may be caused by the staining procedure itself (e.g., insufficient penetration of the indicator dye in all areas), this could also indicate damage resulting from mechanical stress applied when taking the biopsy, despite the all possible precautions, which were taken. However, it is also known that cells and distinct structures of an organ respond differently to stress. In the case of IR, damage to cells, which are central to organ function, is most critical. Often, these cells are metabolically highly active and thus affected in more dramatic ways by a decrease in nutrients and oxygen availability. In line with this and previously published reports [25], we also observe a high degree of heterogeneity regarding the mitochondrial membrane potential in kidney tubules. This is known to be higher in the distal when compared to the proximal tubules [25], the latter being especially vulnerable to mitochondrial dysfunction [26,27]. Interestingly, WGA staining showed an inverse pattern when compared to the TMRM staining, with the proximal tubules being strongly and the distal tubules only weakly stained. Such differences might be explained by differences in the cell membrane carbohydrate composition reported before [28].

Optimizing kidney preservation is one of the main topics, especially in the context of new donor pools such as expanded criteria donors or donation after cardiac death. There are several strategies such as preconditioning of the kidney or improvements in organ preservation solutions which aim to prevent tissue injury [29]. Relevant models and readout systems monitoring the success of such

strategies are the *conditio sine qua non* for such strategies before they can be incorporated into clinical practice. Biopsychronology is suited for such a purpose as it provides real-time spatial information on cell viability and tissue integrity in renal biopsies. For the moment, we only provide proof for the potential of such an approach, which is also suited to describe heterogeneity of the organ. However, translation of the approach to clinic requires intensive testing of the human samples, which would be a part of follow-up study and may help to set criteria based on which one organ could be discarded and another may be transplanted. Future studies will also address the issue of heterogeneity in detail and its correlation with organ function. In addition, we plan to expand this method to other organs.

Authorship

DF, WS, FA, PH: designed the study and contributed important reagents. JT: designed the study, analyzed data and wrote paper. MIA, MH: designed the study, performed research, collected data, analyzed data and wrote paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Movie S1. Murine kidney biopsy stained immediately with PI (red) and Syto 16 (green). For a period of 15 minutes, every minute a z-stack consisting of 6 planes was acquired using 40× water immersion objective.

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