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Acute Kidney Injury Model Induced by Cisplatin in Adult Zebrafish

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TITLE:

Acute Kidney Injury Model Induced by Cisplatin in Adult Zebrafish

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KEYWORDS:

Kidney, Acute Kidney Injury (AKI), Cisplatin, Zebrafish, Inflammation, Flow Cytometry, TUNEL, Cell Death

SUMMARY:

This protocol describes the procedures to induce Acute Kidney Injury (AKI) in adult zebrafish using cisplatin as a nephrotoxic agent. We detailed the steps to evaluate the reproducibility of the technique and two techniques to analyze inflammation and cell death in the renal tissue, flow cytometry and TUNEL, respectively.

ABSTRACT:

Cisplatin is commonly used as chemotherapy. Although it has positive effects in cancer-treated individuals, cisplatin can easily accumulate in the kidney due to its low molecular weight. Such accumulation causes the death of tubular cells and can induce the development of Acute Kidney Injury (AKI), which is characterized by a quick decrease in kidney function, tissue damage, and immune cells infiltration. If administered in specific doses cisplatin can be a useful tool as an AKI inducer in animal models. The zebrafish has appeared as an interesting model to study renal function, kidney regeneration, and injury, as renal structures conserve functional similarities with mammals. Adult zebrafish injected with cisplatin shows decreased survival, kidney cell death, and increased inflammation markers after 24 h post-injection (hpi). In this model, immune cells

infiltration and cell death can be assessed by flow cytometry and TUNEL assay. This protocol describes the procedures to induce AKI in adult zebrafish by intraperitoneal cisplatin injection and subsequently demonstrates how to collect the renal tissue for flow cytometry processing and cell death TUNEL assay. These techniques will be useful to understand the effects of cisplatin as a nephrotoxic agent and will contribute to the expansion of AKI models in adult zebrafish. This model can also be used to study kidney regeneration, in the search for compounds that treat or prevent kidney damage and to study inflammation in AKI. Moreover, the methods used in this protocol will improve the characterization of tissue damage and inflammation, which are therapeutic targets in kidney-associated comorbidities.

INTRODUCTION:

The kidneys are responsible for several important physiological functions that maintain homeostasis, such as blood filtration, removal of excess residues, and regulation of ion concentrations[1]. Damage of renal tissue can lead to a heterogeneous condition called Acute Kidney Injury (AKI), which clinically is described as a rapid decrease in renal function caused by destruction and death of tubular epithelial cells, endothelial cell injury, and leukocyte infiltration [2,3]. AKI is a condition projected to happen in 8-16% of hospital admissions[4], with a high mortality rate that ranges from 20 to 50% in the intensive care unit (ICU)[5]. This ailment is associated with increased hospital stay and considerable use of financial resources[5]. Etiologic factors include dehydration, shock, infections, sepsis, cardiovascular disease, and nephrotoxic drugs[6]. Nephrotoxicity is defined as a renal injury induced by drugs, causing effects as AKI, tubulopathies, and glomerulopathies[7]. Nephrotoxicity affects two-thirds of ICU patients, as approximately 20% of the drugs prescribed in ICU are considered nephrotoxic[8,9], this includes nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics such as vancomycin and aminoglycosides, and chemotherapeutic agents like methotrexate and cisplatin[7]. Cisplatin is one of the most potent and common chemotherapy drugs, used in the treatment of solid tumors, such as head and neck, testicular, ovarian, and bladder[10]. In the kidney, cisplatin is internalized in the proximal convoluted tube (PCT) through the organic cationic transporter 2 (OCT-2) and in high concentrations binds to the DNA triggering cell death pathways[7,10-12]. The accumulation of this drug in the kidney contributes to nephrotoxicity with death and inflammation[13]. This detrimental side effect affects enormously the life and prognosis of one-third of cancer patients undergoing cisplatin treatment, so is imperative the research of new therapies that can lower the nephrotoxicity without losing the killing effect on cancer cells[10].

Because of this nephrotoxic effect, cisplatin is commonly used as an inductor of AKI in experimental animal models, as described forward. In rodents, the first AKI model induced by cisplatin was reported in 1971[14] but at present, many different protocols have emerged using the dose-dependent and cumulative effects of cisplatin[15]. Thereby, depending on the dosage and number of applications, different grades of severity of kidney injury can be induced[16-21]. The most frequent method consists of an intraperitoneal (i.p.) injection of one dose of cisplatin followed by euthanasia in the following days. In this classic protocol, a single high nephrotoxic dose of cisplatin (10-13 mg/kg in mice and/or 3-8 mg/kg in rats) induces severe histological changes, such as loss of brush border and cell debris inside the tubular lumen, a few days after cisplatin injection. The severity of histological changes is dose-dependent, and signs of regeneration are

observed 7 days after cisplatin injection[16,17].

Although rodent models are well established, we decided to take advantage of the characteristics of another vertebrate, focusing our studies on the zebrafish (*Danio rerio*). This fish has been extensively used for modeling human diseases, because of its small size, external fertilization, high reproduction rates, rapid development, transparency of the embryos and larvae, low maintenance cost, similar anatomy to mammals (with some exceptions), high tissue regeneration capacity, social behavior, 70% of genetic similarity with humans and 84% of with human diseases-associated genes[22]. Streisinger et al.[23-25] started the studies with zebrafish that confirmed the practicability of utilizing this model organism for the genetic analysis of vertebrate development. In kidney research, the zebrafish has emerged not only in developmental studies but also as a genetic tool in the search for new genes linked to kidney conditions[26]. Furthermore, the capacity of regeneration without scar formation and the ability to generate nephrons through their life, called neonephrogenesis, make the zebrafish a key animal model for regeneration research[27,28]. Moreover, the availability of experimental models for different kidney illness, including acute and chronic kidney injury, demonstrate the versatility of this experimental organism[26,29]. As in mammals, the renal progenitors of the zebrafish are derived from the intermediate mesoderm. Such renal progenitors generate the pronephros that will later develop to the mesonephros, which will be maintained as a mature organ until adulthood[29,30].

The adult zebrafish kidney is located on the dorsal wall of the body, between the swim bladder and the backbone[29]. From a ventral view, the zebrafish can be segmented into three regions (**Figure 1A**): head (H), trunk (Tr), and tail (Ta)[29]. Same as mammals, the zebrafish has the nephrons as functional units of the kidney, which are divided into tubule segments (**Figure 1A**): renal corpuscle (RC), proximal convoluted tubule (PCT), proximal straight tubule (PST), distal early (DE), late distal (DL) and collecting duct (CD)[29]. Zebrafish shares genetic conservation and structural similarities with human nephrons (**Figure 1B**) but lacks some conformations such as the intermediate tubule, also known as the loop of Henle (LH)[29,31]. Freshwater fishes like zebrafish are normally surrounded by a medium with very low osmolarity, because of this, they tend to be hyperosmotic and depend on the gills, the skin at early stages, and the kidney to regulate osmolarity and water excretion[32]. The filtration of blood from the dorsal aorta by the pronephros begins around 48 h post-fertilization (hpf)[33,34]. The kidney of the zebrafish is not only a metabolic waste excretion organ but also works as a hematopoietic organ from 4 days post-fertilization (dpf) to adulthood and it is equivalent to the bone marrow in mammals[35]. During development, hematopoietic stem cells (HSCs) will seed the kidney, self-renovate, and generate myeloid, erythroid, and lymphoid cell lineages, maintaining transcription factors, signaling molecules, and highly conserved genetic programs with mammals[36,37]. Studies have revealed that most erythroid, thrombocytic, myeloid, and lymphoid cells of the human immune system are present in zebrafish[37,38]. The unique characteristics of this animal and the conserved features with the human kidney made this model organism advantageous in the research of kidney function, injury, and regeneration.

Although the kidney of the zebrafish is well studied and some models of AKI are already available in larva and adult zebrafish[28], at the time of the establishment of this protocol there was no

evidence of a chemically-induced non-antibiotic AKI model in adult zebrafish. Besides this, our laboratory focuses on testing probiotic bacteria and microbiota-derived compounds to study regeneration and renal damage, thus we concentrated our efforts in creating a new cisplatin-induced AKI model in adult fishes. The video article presented in this manuscript demonstrates the procedures for a new model of AKI induction using an i.p. injection of 120 ug cisplatin per g of animal (120 µg/g) (**Figure 2A**). This dose was initially based on studies of AKI induced by cisplatin in murine models that went around 10 mg/kg (equivalent to 10 µg/g)[14-17], however, this dose was. not sufficient to induce kidney damage related to nephrotoxicity (data not shown). Thus, we increased the dose to the ones used in this study (**Figure 2B**). Our work revealed a dose-dependent effect of cisplatin in survival rate after injection with induction of kidney tissue damage 24 hpi as shown by loss of tubular structure, increased inflammatory infiltrate, and high rate of cell death. Here, we describe two techniques for analyzing the development of cisplatin-induced AKI: flow cytometry, to analyze cell infiltration, and TUNEL, to measure cell death. Flow cytometry is a technology that measures the physical (size and granularity) and chemical (fluorescent compounds) characteristics of the cells. Inside the cytometer the cell suspension runs through a sheath fluid that organizes the cells in a single line, allowing them to pass through a laser beam one cell at a time (**Figure 3A**). A detector in front of the light beam will measure the Forward Scatter (FSC), which correlates with cell size, and detectors to the side will measure the Side Scatter (SSC) that correlates to the granularity of the cells. Other detectors will measure the fluorescence from particles, fluorescent-proteins, or antibody-labeled cells[39,40]. As commercial antibodies for zebrafish are scarce nowadays, the use of animal reporters and fluorescent biomarkers allows to improve this analysis and identify diverse cell populations[41-43]. Another tool used in this protocol was the Terminal deoxynucleotidyl transferase (TdT) dUTP Nick End Labeling (TUNEL) assay. The TUNEL assay is a late-stage apoptosis detection method that relies on the ability of the TdT to identify fragmented DNA and label it with deoxynucleotides tagged with a fluorescent marker that later can be visualized and quantified by microscopy[44] (**Figure 3B**)**.** Considering that one of the most striking features of AKI is the induction of apoptosis in tubular kidney cells[3], this technique is extremely advantageous since it can be analyzed by flow cytometry and/or microscopy.

The approaches presented in this article allows the observation of the AKI status and offer a new acute model to study AKI disorders that can be useful for the research of new therapeutic targets in cisplatin-related AKI.

PROTOCOL:

The procedures described in this protocol were previously approved to be used in the zebrafish model by the Animal Use Ethics Committee of the Institute of Biomedical Sciences of the University of São Paulo.

1. AKI Induction by Cisplatin Intraperitoneal Injection

1.1. Prepare **cisplatin working solution** by diluting the stock solution to 850 µg/mL in 0.9% NaCl. Keep at room temperature, protected from light.

CAUTION: The fabricant recommends the manipulation of cisplatin with personal protective equipment (PPE) including goggles, gloves, and lab coat. Store the stock solution at room temperature, protected from light.

1.2. Prepare 150 mg/L MS-222 (Tricaine) anesthetic in system water[45]. Anesthetize adult zebrafish (5-9 months) by immersion for approximately 1-2 min.

NOTE: Effectively anesthetized fish should be irresponsive to touch. To test effective anesthesia, gently press the caudal fin to observe reaction.

CAUTION: Tricaine is an irritant to the skin and eyes, use PPE to manipulate.

1.3. Using a plastic spoon transfer the fish to an absorbent surface, such as paper towels, to remove excess water around the body. Then, with the plastic spoon transfer the fish to a Petri dish over a scale and weigh the fish. Take note of the weight of the fish as it will be necessary for dose calculations.

NOTE: Absorbing excess water from fish prevents overestimate the animal's weight, do not over-dry since is prejudicial to the fish.

1.4. To achieve the **final dose of 120 µg/g** of weight, divide the **µg of the final dose** (120 µg) per the **µg of the cisplatin working solution** (850 µg) and convert this number to microliters (µL) by multiplying for 1000, to get the volume of 120 µg of cisplatin (141.2 µL). Then multiply this number (141.2 µL) for the weight of the fish (g) to get the final volume to be injected.

1.5. With a plastic spoon, transfer the fish on a wet sponge with a little cut to hold it, with the ventral side up. The sponge should be wet with anesthetic in system water.

1.6. Fill a 31 G 1.0 mL insulin syringe with the calculated volume of **cisplatin working solution.**

1.7. Insert the needle into the animal's intraperitoneal portion near the pelvic fin, at a shallow angle to avoid puncturing the viscera (**Figure 2A**). Then slowly inject the solution.

1.8. After injection place the fish in a tank to recover from anesthesia. Watch the fish for signs of normal recovery (*e.g.,* swimming movements, opercular movements).

NOTE: The animal should recover in the next 3-5 min. If necessary, stimulate the fish by moving it with a plastic spoon, a Pasteur plastic pipette, or putting it close to a hose with bubbles.

1.9. For control fish, perform the same procedure by injecting a solution of 0.9% NaCl. Use the same calculation following the proportion of body weight: the volume to be injected will be 141 µL multiplied by the weight of the fish (g).

1.10. Monitor survival of fish at least twice a day in the following days (**Figure 2B**).

2. **Kidney Isolation and Tissue Processing for Flow Cytometry of Immune Cells**

2.1. For this procedure, use immune cell-fluorescent marked transgenic animals (*e.g.*, Tg (*mpo:GFP*)).

2.2. After 24 hpi of 120 µg/g cisplatin, euthanize animals by hypothermal shock (rapid chilling).

NOTE: Hypothermal shock has been demonstrated to be more effective as euthanasia method than MS-222 overdose. Hypothermal shock is less stressful, fast, consistent, and safer for personnel than the use of MS-222, has previously described[46,47].

2.3. In an outer crossing tank prepare ice-water in a 5:1 ratio of ice to system water, put the inner tank with a screen over the ice, wait until water reaches 2-4 °C.

NOTE: Fishes should not be in direct contact with the ice, because this may cause thermal burns and pain.

2.4. Transfer animal to ice-water, wait at least 10 min until there is loss of orientation and no opercular movement.

2.5. With a plastic spoon, place the fish on paper towels to dry off the excess water.

2.6. Transfer the fish to a 3% agarose dissection plate and take it under a stereoscope with upper light. With scissors, decapitate fish making a fast cut just behind the eyes, and remove the head.

2.7. With fine scissors make a cut from the open side to the cloaca and remove the internal organs with fine forceps.

2.8. Use insect pins to pinch the sides of the body walls to open the carcass and expose the kidney attached to the backbone.

2.9. Detach the kidney with fine forceps and place the organ in a 6 well plate with a cold solution of 1x PBS/2% FBS. Keep on ice.

2.10. Pick up the tissue with a Pasteur plastic pipette and pass the tissue through a 40 µm cell strainer over a 50 mL tube, gently macerating it with a syringe plunger.

2.11. Wash twice with 1 mL of 1x PBS/2% FBS and collect the cells in a 50 mL tube.

2.12. Centrifuge cells at 400 × g for 5 min at 4 °C.

2.13. Carefully pick up all the supernatant with a 1 mL micropipette and discard it. Add 500 µL of cold 1x PBS to resuspend the cells and place them in 5 mL flow cytometry tubes. Keep on ice.

2.14. Count cells in Neubauer chamber making a 1:10 dilution in Trypan Blue (*e.g.*, take 10 µL of the sample and mix with 90 µL of Trypan Blue). Add 10 µL of the mixture to a Neubauer chamber and count cells under the microscope.

NOTE: Optimal results are expected with 1-5 x 10^6 cells/mL and >80% viability.

CAUTION: Trypan blue is a carcinogenic agent, use PPE to handle.

2.15. Take the cells to be read by a cytometer. Then analyze the results selecting the population of interest.

3. Processing of Adult Zebrafish Kidney Tissue for TUNEL Assay

3.1. For this procedure, use wild-type animals (*e.g.*, AB, Tübingen, etc.) or a transgenic animal with different fluorescent color than the TUNEL kit, as similar fluorescence can interfere with TUNEL's analysis.

3.2. After 24 hpi of 120 µg/g cisplatin, euthanize animals by hypothermal shock (rapid chilling). See 2.3-2.4.

3.3. Dissect the fish as described in 2.5-2.6; the kidney must remain attached to the backbone during the fixation procedure (explained below).

3.4. Using insect pins, pinch the sides of the body walls to open the carcass and pin it on a cork surface to keep the kidney exposed.

NOTE: This procedure ensures that the kidney remains in the right position for later analysis.

3.5. Then place the cork surface with the kidney facing down in a 6 well plate over a freshly made solution of 4% paraformaldehyde (PFA). Keep it overnight at 4 °C.

CAUTION: PFA is carcinogenic and irritant for skin and mucous surfaces. Prepare PFA solutions under a chemical hood using PPE including eye protection equipment.

3.6. The next day, dissect the kidneys as in 2.8. Place the kidneys in a 60 mm Petri dish with 1x PBS and rinse twice in 1x PBS.

3.7. Prepare 2% agarose to generate a support matrix for the tissue.

3.8. Discard all remaining 1x PBS from the Petri dish and pour 2% agarose slowly to cover the whole organ. Then position the kidney using fine forceps under a stereomicroscope to prevent

the kidney to fold. Let agarose solidify at room temperature.

NOTE: This procedure will keep the orientation and shape of the organ through histological processing since the leaf-like shape of the organ causes a tendency to fold if it is not inside a supportive matrix.

3.9. After agarose solidification, use a scalpel to cut the agarose around the tissue, forming small cubes, and remove the excess of agarose around the tissue.

3.10. Place the agarose cubes in a cassette suitable for histological processing.

NOTE: The following steps can be done manually or in an automatic tissue processor.

3.11. First, process the tissue in the cassette following the next steps for 45 min each at room temperature: one bath of 50% ethanol, one bath of 70% ethanol, two consecutive baths of 95% ethanol, and three consecutive baths of 100% ethanol. Afterward, process the tissue in two consecutive baths of Xylene and three consecutive baths of paraffin; the latter lasts 1 hour each at 60 °C.

CAUTION: Make changes under a chemical hood, vapors from ethanol and xylene are irritant and toxic.

3.12. To prepare paraffin blocks, melt paraffin lentils to 60 °C.

3.13. Open the plastic cassette with the tissue inside and keep it on a warm plate. Warm-up metal molds for the paraffin.

3.14. With tweezers place the tissue over a metal mold so that the kidney length is parallel to the mold base. Add the paraffin, reallocate the tissue if necessary.

3.15. Cover the mold with the base of the cassette and add paraffin until the grid is covered. Let solidify at room temperature and then place at -20 °C for a faster solidification process.

3.16. Release paraffin block from the metal mold around 20-30 min later.

3.17. With a microtome, section the tissue embedded in paraffin to 5 µm thickness. Use silanized or positively charged glass slides to collect the tissue.

4. TUNEL assay

NOTE: The following protocol uses an In Situ Cell Death Detection Kit (Table of Materials).

4.1. Dewax tissue slides placing them in two consecutive baths of xylene for 5 min. Then rehydrate the tissue through a graded series of ethanol: 100%-95%-70%-50%, for 5 min each.

4.2. Place slides in running cold tap water to rinse off the ethanol. Keep the slides in distilled water.

4.3. Prepare a dark incubator chamber. Add wet paper towels on the bottom to keep the moisture during the incubation steps.

NOTE: In lack of an incubator chamber is possible to use a Petri dish with moist paper in the bottom and two toothpicks to place the slide.

4.4. Prepare fresh **Proteinase K working solution**: 10 µg/mL in 10 mM Tris/HCl, pH 7.4-8.

NOTE: Proteinase K is used as a permeabilization agent, as recommended by the fabricant.

4.5. Place the slides in the dark incubator chamber and add **Proteinase K working solution** until cover samples. Incubate for 30 min at 37 °C.

4.6. While samples are incubating, prepare the **TUNEL reaction mixture**: Add 50 µL of Enzyme Solution to 450 µL Label Solution. Protect from light.

NOTE: The volume to be prepared can be adjusted in the same 1:10 proportion. The volume is calculated to be 50 µL of the mixture for each section; this can change depending on the size of the samples.

4.7. Pick up the dark chamber and wash the slides twice with 1x PBS.

4.8. Next, dry the region around the sample using absorbent paper and add 50 µL of **TUNEL reaction mixture** over each tissue slide, spread the solution so that the whole sample is covered. Incubate at 37 °C for 2 h. Protect from light.

4.9. After incubation, rinse the slide three times with 1x PBS and dry the region around the sample using paper towels.

4.10. Add 50 µL of DAPI 1:1000 to the samples, for nuclear counterstaining, and incubate for 5 min at room temperature. Protect from light.

4.11. Rinse again three times with 1x PBS and dry the region around the sample.

4.12. Mount the slide with an anti-fade hydrophilic medium, place a coverslip, and seal with nail polish. Store slides horizontally, protected from light at 4 °C.

NOTE: The anti-fade properties of the mounting medium are to preserve the fluorescence of the samples but is possible to use any hydrophilic medium available. The final sealing step with nail polish is crucial to avoid dehydration.

4.13. Visualize the samples under a fluorescence microscope. For this type of fluorescent pigment, use an excitation wavelength in the range of 520-560 nm (green) and detection in the range of 570-620 nm (red).

REPRESENTATIVE RESULTS:

The kidney of the zebrafish is a flat pigmented organ located on the dorsal wall and its basic functional unit, the nephron, is conserved with mammals (**Figure 1**)**.** The particularity of having just one kidney with a high capacity of regeneration makes this model organism an excellent choice for model kidney injury. The protocols presented in this work are designed to induce AKI by the intraperitoneal (i.p.) injection of cisplatin in adult zebrafish (**Figure 2**) and to later be analyzed by two techniques detailed before: flow cytometry (**Figure 3A**) and TUNEL (**Figure 3B**). A flowchart of the whole process is depicted in **Figure 4**. The doses of cisplatin were applied based initially on those described in mouse models[15-17], in which the standard used is 10-13 mg of cisplatin per kg of animal (mg/kg). However, the zebrafish showed to be more resistant to cisplatin than the mouse (data not shown), and the final dose was increased. When we evaluated the survival rate of the animals, the experiments showed a dose-dependent effect of cisplatin (**Figure 5A**). Because of this, we recommend following the instructions exactly as explained in this protocol and monitor the survival rate of the animals constantly as a measure of reproducibility, before collecting any material. After the i.p. injection of 120 µg/g cisplatin (**Figure 5A, red line**), a decrease in survival of around 30% of the animals was observed in the first 24 h and the survival decreased gradually until reaching around 20% of live animals on day 5 post-injection, then stabilized (**Figure 5A**). Cisplatin toxicity was not affected by the sex of the animals, since males and females have similar survival curves (**Figure 5B**).

Analysis of the kinetics of cisplatin-induced AKI showed increased inflammation and cell death in the kidney 24 hpi. One of the fastest and quantitative ways to evaluate inflammation is flow cytometry but given the lack of antibodies against zebrafish antigens available commercially for this technique, is necessary to use a transgenic line with an immune marker. Nowadays, many zebrafish lines labeling immune cells are accessible (**Table 1**). These lines can be singly used or in combination, given enough repertoire for analysis[48-60]. This simplifies enormously the technique since is not necessary any antibody incubation step, on the contrary, after the isolation of the cells by mechanical separation, the direct reading on the cytometer is possible.

As mentioned before, the kidney of the zebrafish is not only a blood filtration organ with homeostatic functions but also the anatomical site of hematopoiesis in adults, equivalent to the bone marrow in mammals[33-35]. This way when we analyze it by flow cytometry is possible to differentiate cell populations comparable to the human blood[61,62] (**Figure 6A**), this allows us to identify the cell populations initially by size and granularity and exclude debris. In this case, we used a transgenic line called *Tg(mpo:GFP)*[52] that expresses a green fluorescent protein (GFP) together with the enzyme myeloperoxidase, which is present in neutrophils. Knowing this, our gate strategy was based on the initial separation of the granulocyte's population (**Figure 6B**)**.** Following this, doublet cells were excluded, since they can significantly alter the analysis and lead to inaccurate conclusions. A doublet is a single event that consists of 2 independent particles and

can be excluded by selecting a forward scatter height (FSC-H) vs. a forward scatter area (FSC-A) density plot (**Figure 6C**)**.** After this step, the cells that expressed the fluorescent marker were identified and selected (**Figure 6D**). Finally, the population statistics were extracted from the analysis and plotted as percentage of cells (**Figure 6E**)**.**

One of the most prominent characteristics of cisplatin nephrotoxicity is tubular cell death[10], and to easily visualize this we used the TUNEL assay for apoptosis detection. This method recommends using wild-type cells and tissues that lack fluorescent markers, since parallel fluorescence would interfere with the analysis, in the case of the zebrafish is recommended to use wild-type lines, such as AB, Tübingen, TAB, or a transgenic line with a fluorescent protein that does not interfere with the TUNEL fluorescence color. The TUNEL technique allows the analysis via flow cytometry or microscopy. Microscopy has the advantage of conserving the tissue structure, allowing to see which cells are dying. Under the fluorescent microscope, the bright nuclei of apoptotic cells can be easily differentiated from the background. Animals injected with cisplatin (**Figure 7B**) have more dead cells than the control (**Figure 7A**) at 24 hpi. The final quantification was made with the cell-counter option of FIJI Software and showed statistically more dead cells in cisplatin-treated kidneys than in the controls (**Figure 7C**)

The protocol described in this manuscript showed how to use cisplatin as an inducer of AKI in adult zebrafish, which is dose-respondent, fast, and reliable. Based on the data obtained from survival rates and the measurement of signs of nephrotoxicity of cisplatin including inflammation (detected by flow cytometry) and cell death (detected by TUNEL assay), we propose this model for the study of cisplatin nephrotoxicity as well as for future treatments in AKI-related diseases.

FIGURE AND TABLE LEGENDS:

Figure 1: Structure and comparison of zebrafish and human kidneys. A. (1) Lateral view of an adult zebrafish with the kidney represented in dark brown located in the dorsal wall of the fish, between the swim bladder (sb) and the backbone. (2) Ventral view of the kidney showing nephrons (yellow) connected to the collecting duct (blue). The different regions of the kidney are flagged: head (H), trunk (Tr), and tail (Ta). (3) Schematic representing zebrafish nephrons and their segments labeled and colored to match genetic conserved regions with human nephron. **B.** (1) Sagittal view of a human kidney. (2) Schematic depicting a human nephron with segments labeled and colored. RC: renal corpuscle; PCT: proximal convoluted tubule; PST: proximal straight tubule; TL: thin limb; LH: Loop of Henle; TAL: thick ascending limb; DE: distal early; DL: distal late; DCT: distal convoluted tubule; CD: collecting duct.

Figure 2: Experimental design for cisplatin-induced AKI. A. Lateral and ventral view of adult zebrafish pointing the position of the needle during the injection procedure. The needle penetrates at a 20-30° angle from the belly and is inserted slowly parallel to the ventral wall avoiding puncturing the viscera. **B.** Experimental design of cisplatin-induced AKI: (1) Injection of cisplatin 120 µg/g per animal at day zero. (2) Before attempting step 3, survival monitoring of fishes after injection is recommended from day one until day ten. (3) Kidney dissections one day after cisplatin injection for further processing techniques.

Figure 3: Mechanisms of flow cytometry and TUNEL techniques. A. Overview of the flow cytometer: a suspension of cells is hydrodynamically focused on a single line by a sheath fluid, causing cells to pass one by one in front of a laser beam. Detectors in front and on the side measure the forward scatter (FSC), side scatter (SSC), and fluorescence of the cells. **B.** Principle of TUNEL assay. Terminal deoxynucleotidyl transferase (TdT) mediates the addition of a fluorescent-marked dUTP to 3'-OH ends of a fragmented DNA.

Figure 4: Flowchart of represented techniques. A. A flowchart showing the steps to follow when choosing to analyze the kidney tissue through flow cytometry (orange) or TUNEL (blue), when inducing AKI by cisplatin injection (grey).

Figure 5: Survival monitoring of cisplatin injected fish. A. Survival rate of different dosages of cisplatin injections (25 – 50 – 112.5 – 120 µg/g). Log-rank (Mantel-Cox) test, ** $p < 0.01$. **B.** Survival rate of males vs. females injected with 120 µg/g cisplatin. Log-rank (Mantel-Cox) test, *** $p < 0.001$.

Figure 6: Gate strategy for transgenic zebrafish line. A. Density plot of zebrafish adult kidney cells, populations are separated by size (FSC-A) and granularity (SSC-A). Different populations are selected by colored ovals/circles. Pink: Erythroid; Black: Lymphoid; Yellow: Precursors; Red: Granulocytes. **B.** Density plot of side scatter area (SSC-A) and forward scatter area (FSC-A) for selection of Granulocytes population in the kidney. **C.** Density plot of forward scatter high (FSC-H) and forward scatter area (FSC-A) for selection of singlets population inside the granulocyte gate. **D.** Density plot of forward scatter area (FSC-A) and FITC-A:MPO for selection of *mpo:GFP* positive cells (neutrophils) in the kidney. A positive population is considered around 10^3 on, of fluorescence intensity. **E.** Graph of the percentage of *mpo:GFP* positive cells (neutrophils) in Control vs. Cisplatin animals, 24 hpi . Unpaired *t*-test.

Figure 7: TUNEL assay of cisplatin injected fish. A. Microphotographs of fixed adult kidney 24 h after 120 µg/g cisplatin injection. Controls are injected with 0.9% NaCl. TUNEL positive cells (apoptotic cells) are stained in red (white arrows). DAPI (blue) is used as a nuclear counterstain. Scale bar: 50 µm. 20x magnification. **B.** Graph showing quantification of the number of dead cells in the kidney by 20x field. Unpaired *t*-test, * $p < 0.05$.

Table 1: Zebrafish transgenic lines for immune cells. Table resuming the names of zebrafish reporter lines with the respective type of immune cell labeled and the reference articles where they were constructed. A combination of these zebrafish lines can offer new possibilities of cell selection by flow cytometry.

DISCUSSION:

The prevalence of kidney disease has continued to increase worldwide, becoming a global public health problem that affects millions of people[63]. Finding a way to treat kidney injured individuals is of paramount importance as well as understand more about their etiology and progression. Several studies have been using animal models to understand renal damage. The zebrafish kidney (**Figure 1**) has been studied for years in developmental biology and injury research because of its

self-regenerating capacities and genetic similarity[29,64]. Here, we present a new AKI model in adult zebrafish using the properties of cisplatin as a nephrotoxic agent, detailing the steps for accomplishing a fast and acute reaction with damage visible as soon as 24 hpi (**Figure 2**). Moreover, here we explain two techniques that will help to the evaluation of the tissue damage after the cisplatin injection, flow cytometry and TUNEL (**Figure 3**).

Current AKI models in adult zebrafish include the i.p. injection of gentamicin which induces extensive damage in the nephron and tubule destruction, neonephrogenesis events start from day 5, and regeneration is completed by 21 days post-injection[65]. On the other hand, a model of sepsis-associated acute kidney injury (S-AKI) was established by the infection with *Edwardsiella tarda*, since significantly increased the expression of AKI markers, such as insulin-like growth factor-binding protein-7 (IGFBP7), tissue inhibitor of metalloproteinases 2 (TIMP-2), and kidney injury molecule-1 (KIM-1), in larvae and adult zebrafish[66]. The zebrafish is known for being a high-throughput animal for the search of therapeutic agents and this includes the use of probiotics and microbiota-derived metabolites to study kidney function and regeneration[67]. However, the available models could directly affect the outcome of these treatments. Thus, we established a different method to induce AKI in adult zebrafish (**Figure 4**), using cisplatin as a known nephrotoxic agent that would not have direct known effects on the fish microbiota, as would the gentamicin model for being an antibiotic, or the infection with *E. tarda*, for being a sepsis model. However, at the same time that we were developing our cisplatin protocol, another group also explored the nephrotoxic effects of cisplatin in adult zebrafish, simplifying the dose to 10-20-30 µg per animal[68]. Although they also showed cisplatin dose-dependent effect in survival, we recommend caution in using a single quantity of cisplatin for all fishes, as zebrafish from the same age can have very different sizes and weight and this could induce variations in the results[69,70]. We think is important to adjust the dose to the corresponding weight of the animal, as is done in mice and this study.

In our experiments with adult zebrafish, cisplatin showed a dose-response effect. This was visualized by monitoring the survival rate of the animals after cisplatin injection (**Figure 5**). We used survival as a way of estimate the intensity of the dose of cisplatin and not as a measure of nephrotoxicity, as no other physical sign is visible during the monitoring time. This can be comparable with rodents, in which the severity of the kidney injury can be modulated by the dosage and frequency of cisplatin injection[15], achieving lethal doses with higher concentrations of cisplatin[71]. Dead is also seen in the following days in the larval model of cisplatin[72]. Since our aim was to induce an acute injury in few days, we selected the 120 µg/g dose of cisplatin as is possible to observe kidney damage 24 h after the injection, however, this can be adjusted depending on the objectives of the study.

In humans, AKI is clinically diagnosed by decreased glomerular filtration rate (GFR), elevated serum creatinine, and blood urea nitrogen[3]. In zebrafish, the repertoire of AKI models includes some genetic-conditional models[73,74] and some drug-related models[65,72], but as some of the AKI functional parameters cannot be measured on zebrafish because of technical difficulties (*e.g.,* blood collection), most research adopts morphological and visual techniques to observe the features of AKI[1,75] such as our study.

In rodents, cisplatin enters the epithelial cells in the proximal and distal tubules, inside the cell undergoes metabolic activation and becomes highly reactive acting on cell organelles and inducing changes in cell structure. These changes can induce apoptosis and autophagy and even necrosis, at very high doses. In response to this damage, many cytokines are released and leukocytes are recruited leading to inflammation and affecting the functionality of the organ[15]. This highlights the importance of assessing what type of cells can be found in the injured kidney, as residents or infiltrated immune cells. Here we showed how to assess this by flow cytometry, using the transgenic immune reporter lines available nowadays (**Table 1**). Cisplatin increased the percentage of neutrophils (*mpo:GFP* positive cells) in the kidney 24 h after the injection (**Figure 6**). In the case of the zebrafish, the kidney is the niche of HSCs that give rise to different blood cell types. Nonetheless, many granulocytes and macrophages are normally circulating in the blood. In our example, we used the *mpo:GFP* transgenic line that express GFP under the promoter of myeloperoxidase of neutrophils[52]. Original studies of the *mpo:GFP* transgenic line demonstrated expression of myeloperoxidase in different states of neutrophil maturation[76] but our gate strategy focused on the granulocyte fraction that comprises mature cells coming from the blood[52], this way our analysis include infiltrated cells and not resident cells. This is important to consider when isolating the desired cell population.

As explained above, apoptosis is the most classic marker of cisplatin-related AKI. Here, we demonstrated a simple protocol for the localization of dead cells by the TUNEL assay. Cisplatin injection increased the number of apoptotic cells 24 hpi (**Figure 7**). This can be easily quantified by counting directly the dead cells from the tissue. Nonetheless, for the identification of cell-specific death the use of antibodies against the desired cell (*e.g.,* tubular cells), or the use of a transgenic reporter line can be used together with this technique. When compared with the gentamicin-induced model of AKI, cisplatin seems to be a more severe model, since gentamicin apoptosis was higher on the third day after injection[65].

Despite having a variety of side effects, cisplatin is still widely used in cancer therapy, because of its effectiveness against various types of cancers, including carcinomas, germ cell tumors, lymphomas, and sarcomas[77]. Nephrotoxicity occurs in one-third of patients in treatment with cisplatin[10], thus the search for strategies that can decrease this effect and increase renoprotection is imperative. We believe that the methods and techniques presented in this manuscript will help to elucidate mechanisms of kidney injury and find therapeutic targets that can be essential to improve the quality of life of individuals that suffer from renal complications, predominantly the ones related to the use of cisplatin.

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DISCLOSURES:

The authors have nothing to disclose.

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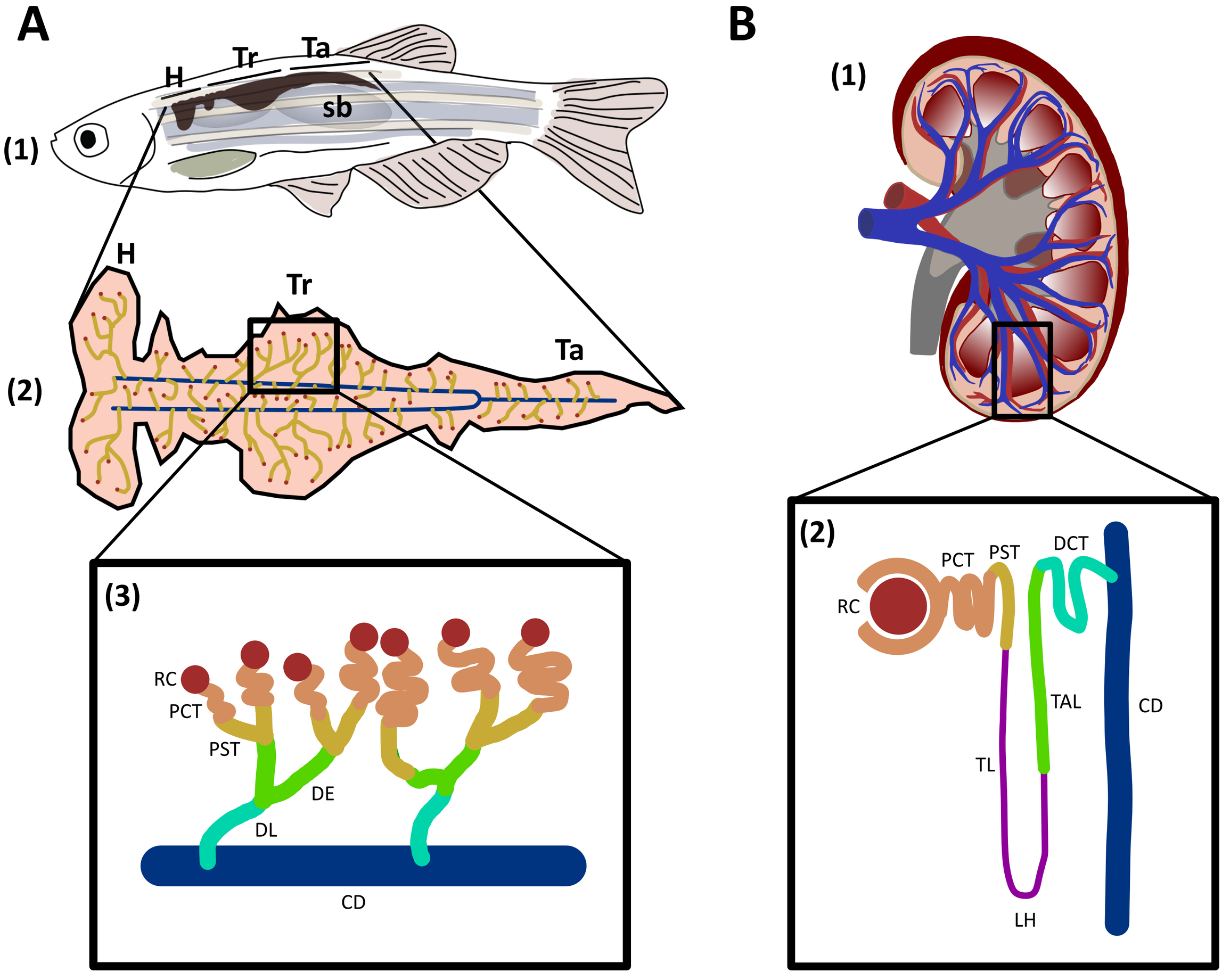

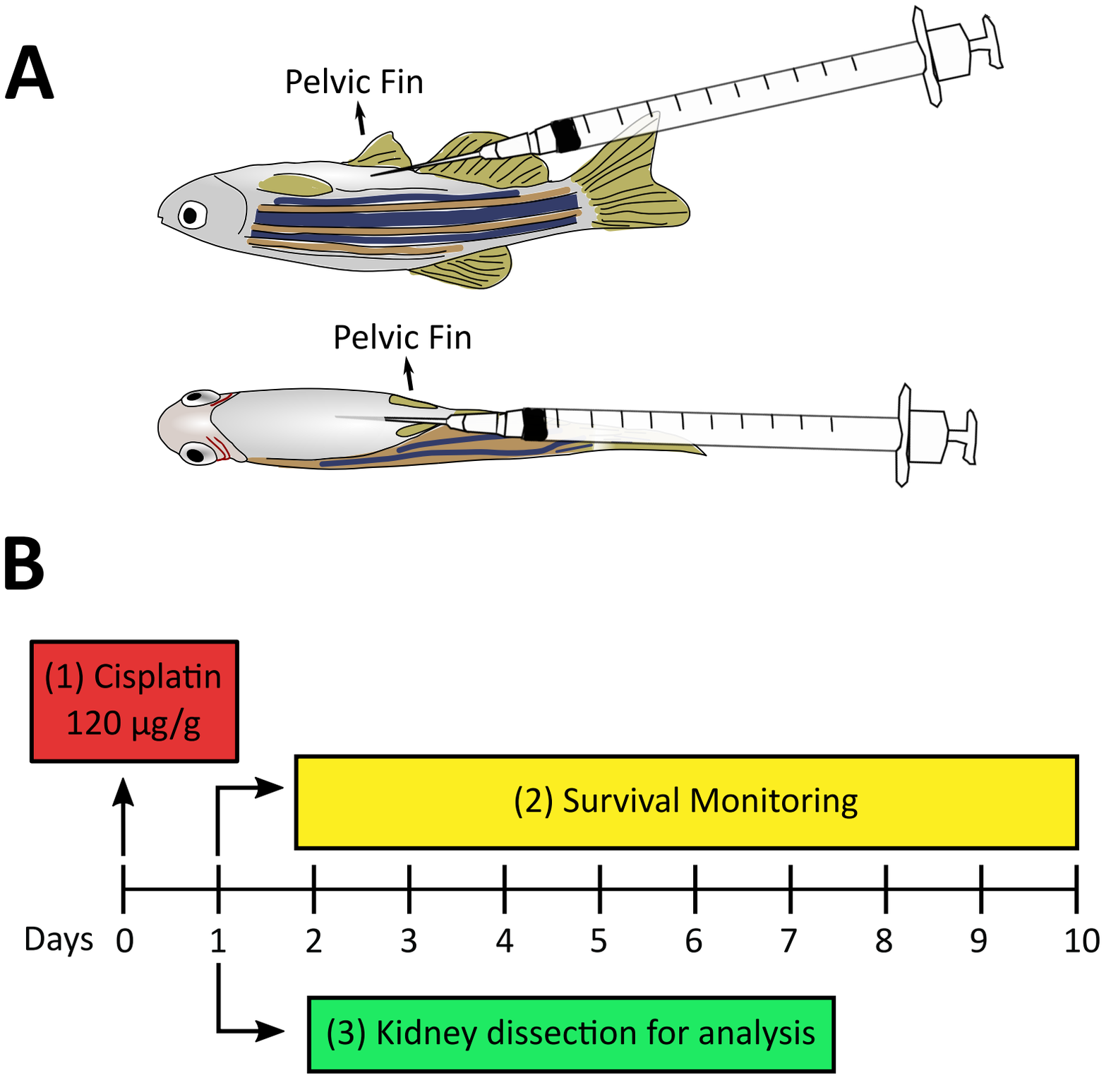

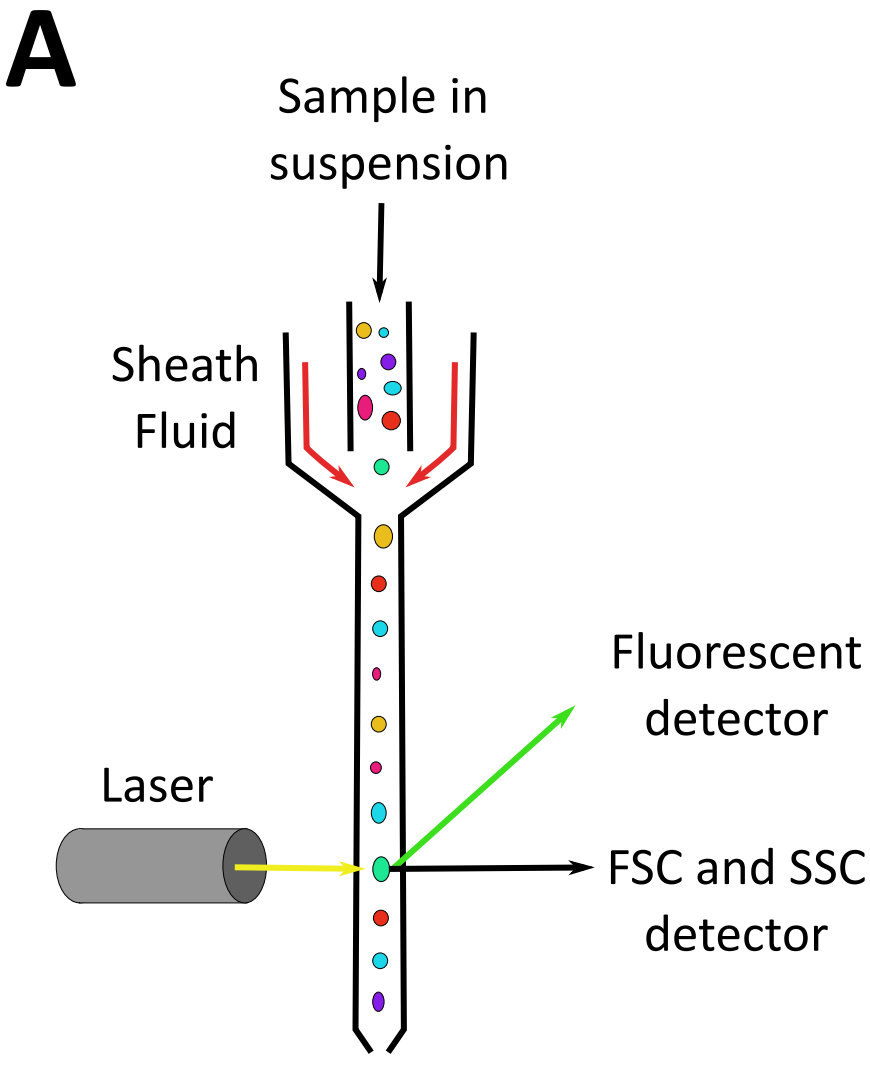

Figure 4

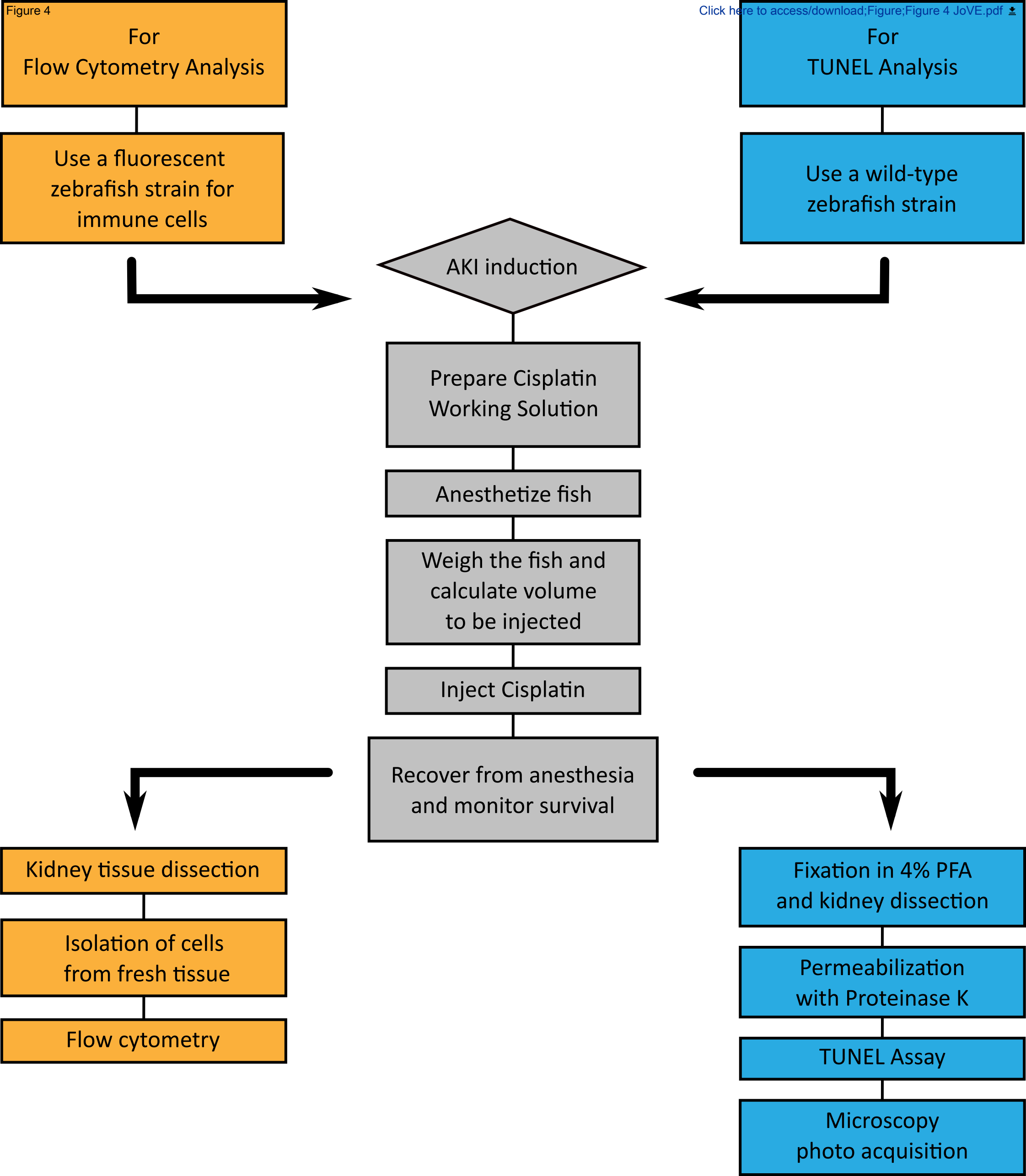

Figure 5

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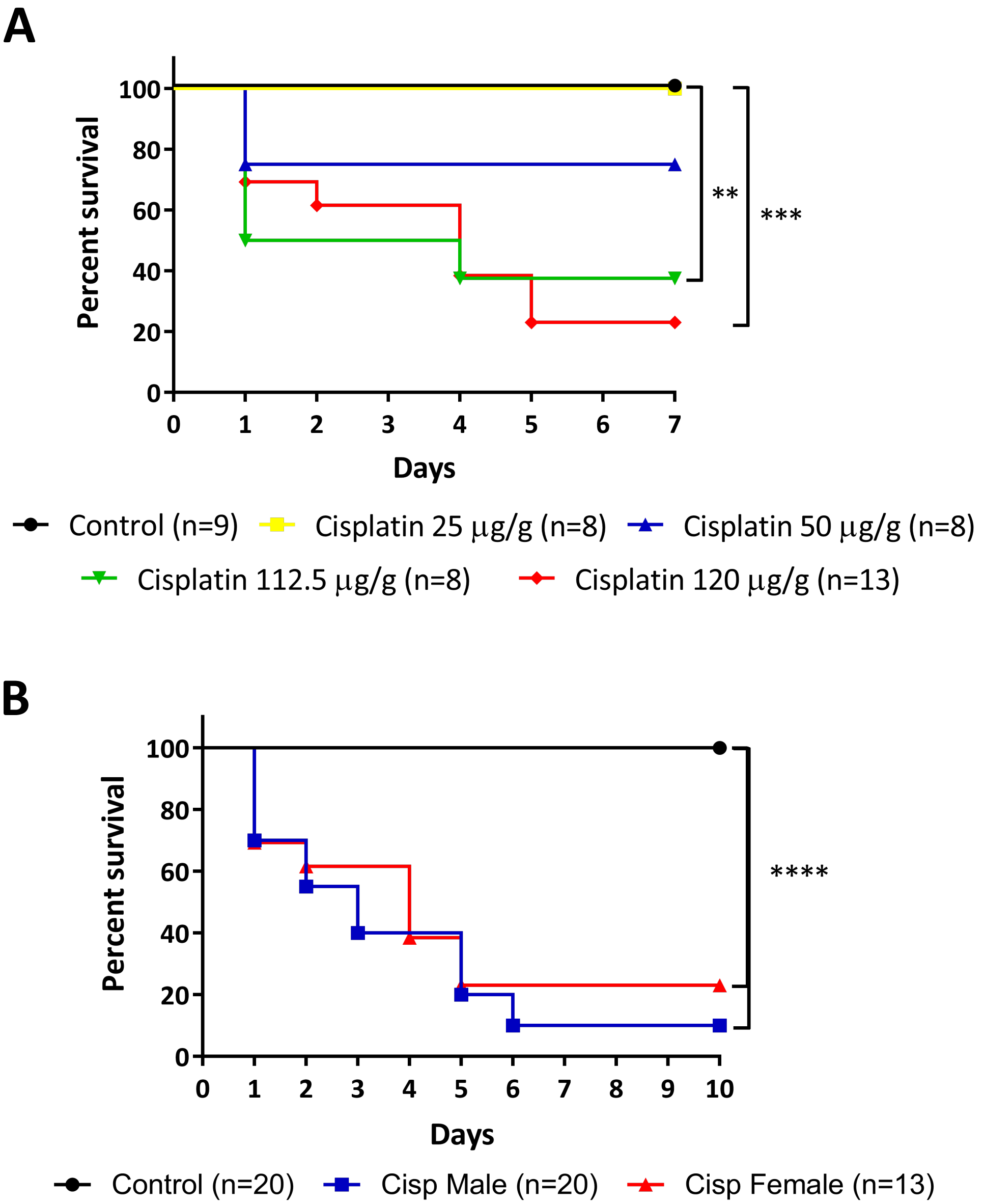

Figure 6

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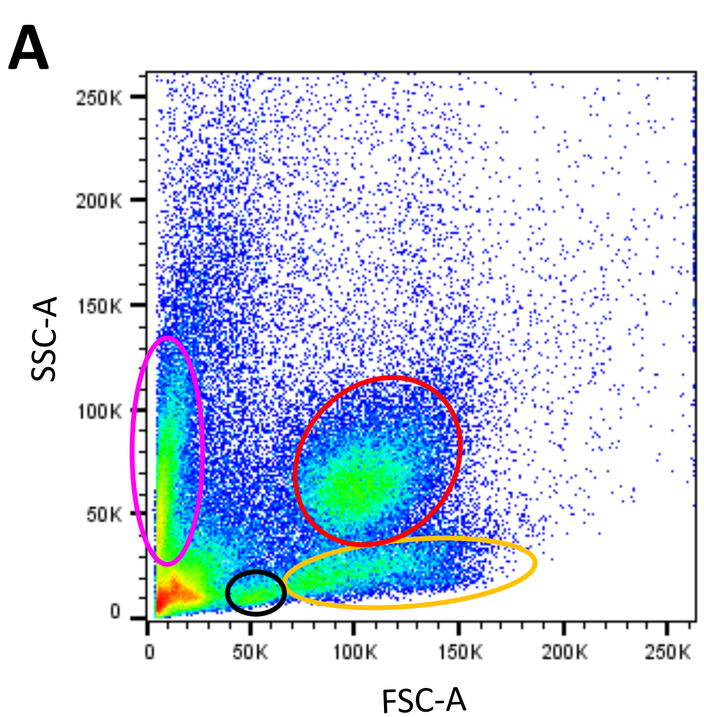

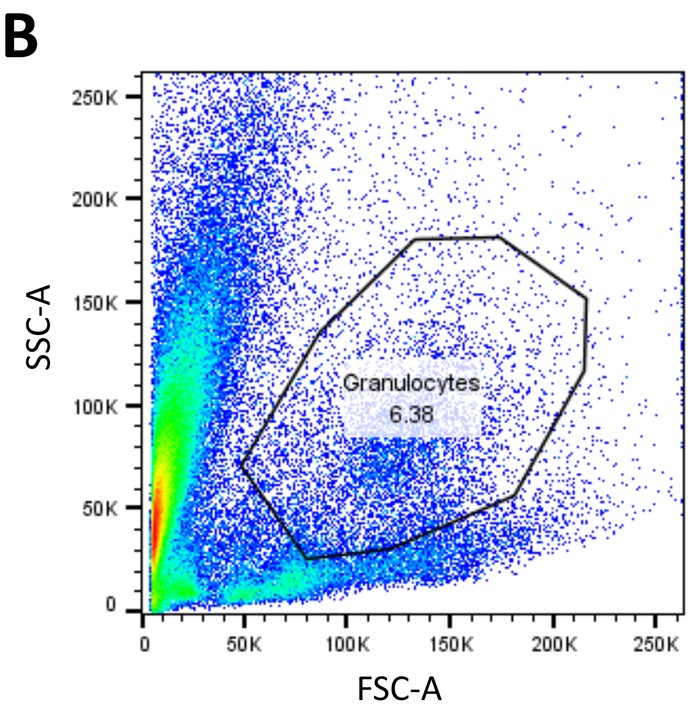

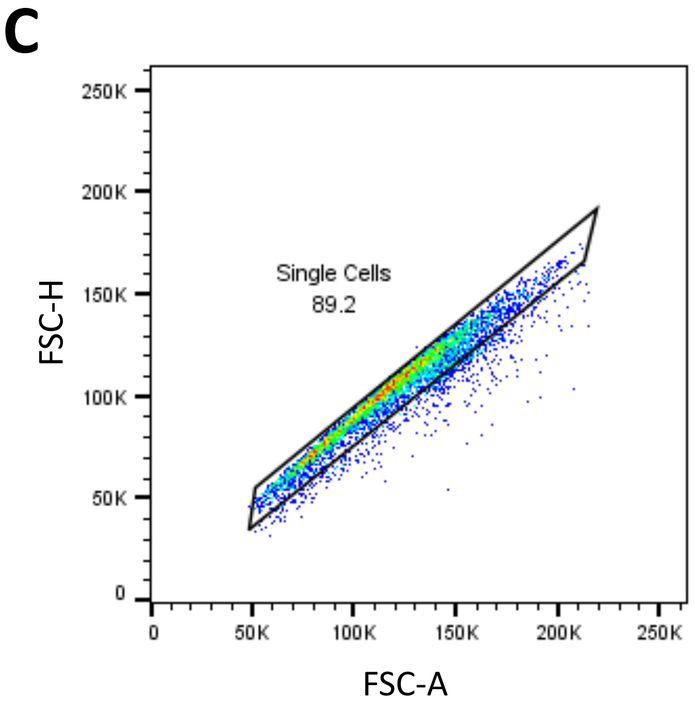

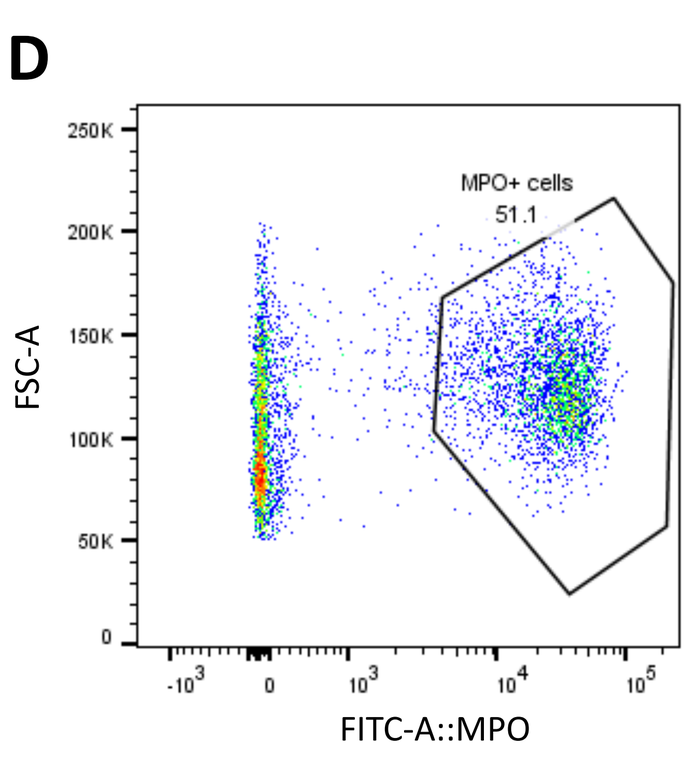

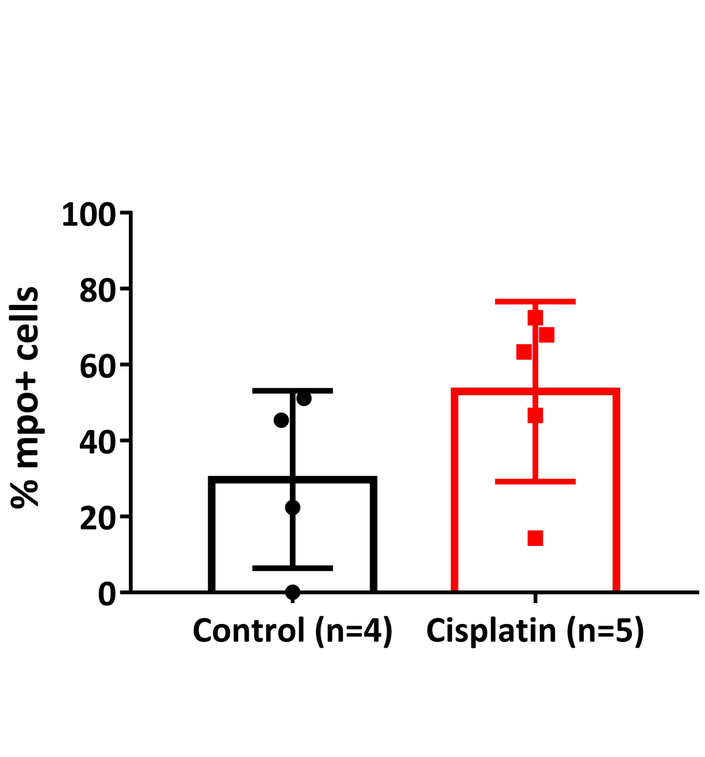

Figure 7

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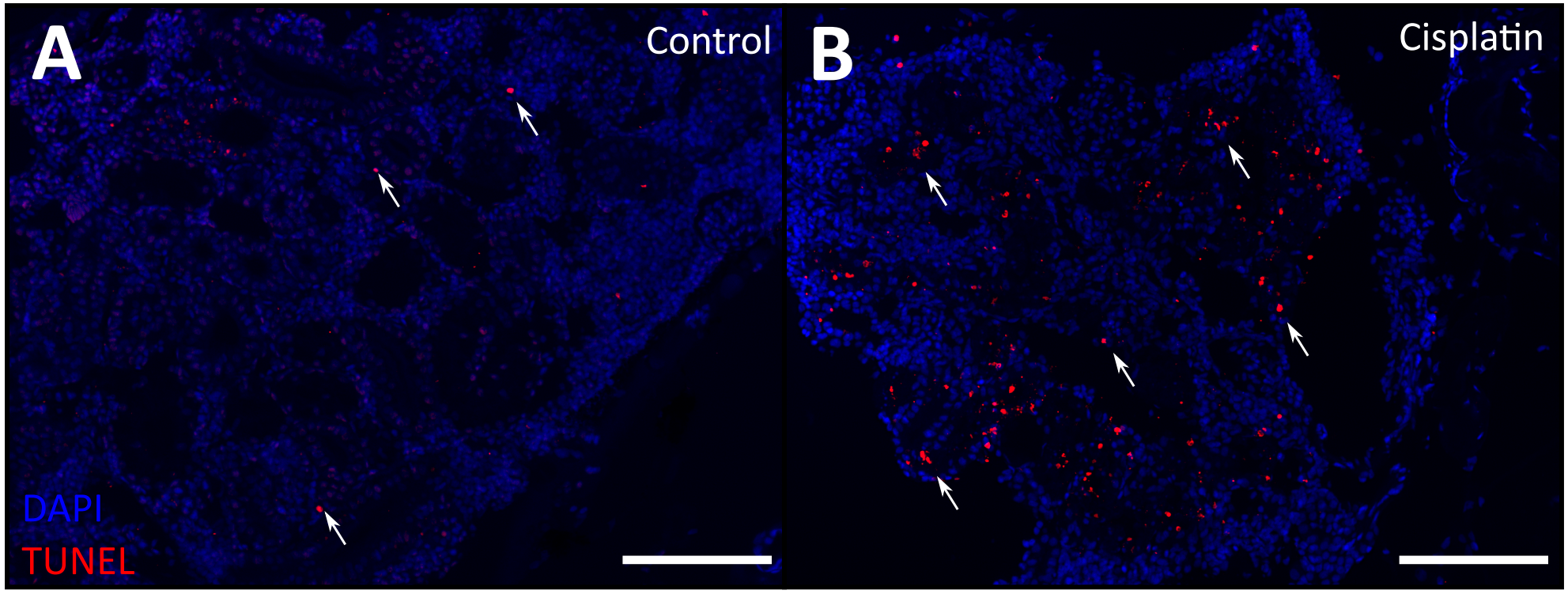

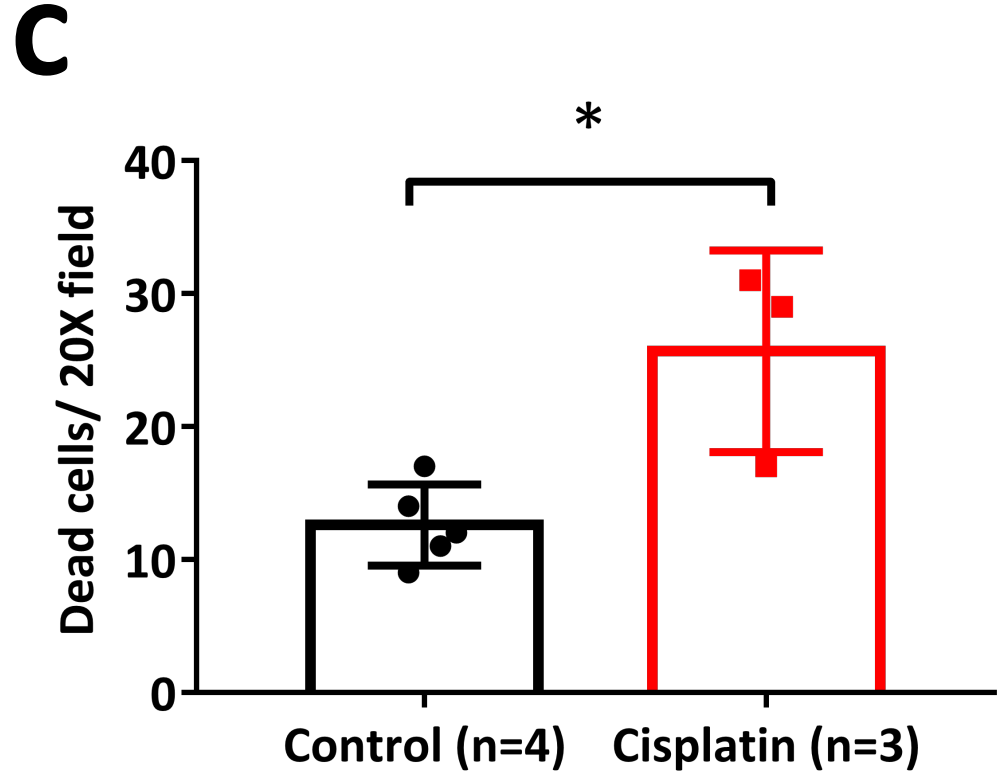

Transgenic Line	Cell Type Labeled
Tg(spi1:EGFP)pA301	Myeloid cells
Tg(zpu1:GFP)	Myeloid cells
Tg(mhc2dab:GFP)sd6	Monocytes
Tg(lysC:DsRED2)	Neutrophils
Tg(mpo:GFP)	Neutrophils
Tg(mpeg1:mCherry)	Macrophages
Tg(mpeg1:Dendra2)	Macrophages
Tg(lck:GFP)	T-cells
TgBAC(ikaros:EGFP)	T-cells
Tg(rag1:GFP)	T-cells
Tg(rag2:GFP)	T-cells
Tg (CD79:GFP)	B-cells
Tg(CD45:DsRed)	Leukocytes

References

Ward *et al.* 2003[48]

Hsu *et al.* 2004[49]

Wittamer *et al.* 201150

Hall *et al.* 2007[51]

Mathias *et al.* 2006[52]

Ellett *et al.* 2011[53]

Harvie *et al.* 2013[54]

Langenau *et al.* 2004[55]

Bajoghli *et al.* 2009[56]

Jessen *et al.* 1999[57]

Jessen *et al.* 2001[58]

Liu *et al.* 2017[59]

Bertrand *et al.* 2008[60]

Name of Material/ Equipment	Company	Catalog Number
1x PBS		
31 G 1.0 cc insulin syringe	BD Plastipak	990256
3.5 L Fish tank	Tecniplast	
6 well plate	Corning	351146
10 mM Tris/HCl		
50 ml Falcon tube	Corning	352070
2-3% Agarose	Invitrogen	16500-500
2% FBS	Gibco	12657-09
4% Paraformaldehyde	Sigma-Aldrich	P6148-500G
50% Ethanol		
70% Ethanol		
90% Ethanol		
100% Ethanol	Synth	00A1115.01.BJ
100% Xylene	Synth	00X1001.11.BJ
Cell strainer 40 µm	Corning	431750
Cisplatin	Blau Farmacêutica	16020227
Cork board sheet		
DAPI	Sigma-Aldrich	D9542
Fine forceps	Fine Science Tools	11254-20
Flow cytometry tubes	Corning	352052
Glass slide	Thermo-Fisher	4445
Histology cassette	Ciencor	2921
Immuno stain chamber	Ciencor	EP-51-05022
Incubator	NAPCO	5400
Insect pins	Papillon	
In Situ Cell Death Detection Kit	Roche Diagnostics	12156792910
Metal mold	Leica Biosystems	3803081
Micropipette 200-1000 µL	Eppendorf	
MS-222 (Tricaine)	Fluka Analytical	A5040-25G
NaCl 0.9%	Synth	C1060.01.AG
Nail polish		

Neubauer chamber	Precicolor HGB	
Pasteur plastic pipet	United Scientific Supplies	P31201
Paraplast	Sigma-Aldrich	P3558
Petri dish	J.ProLab	0307-1/6
Plastic spoon		
Proteinase K	New England BioLabs	P8102
Scissors	Fine Science Tools	14060-09
Scalpel blade	Solidor	
Sponge		
Trypan Blue	Cromoline	10621/07
Vannas Spring Scissors	Fine Science Tools	15000-00
Vectashield Antifade Mounting Medium	Vector Laboratories	H-1000-10
Centrifuge	Eppendorf	5810R
Cytometer	BD Biosciences	FACSCanto II
Fluorescence Stereoscope	Zeiss	Axio Zoom.V16
Fluorescence Microscope	Zeiss	AxioVert.A1
Microtome	Leica	Jung Supercut
Scale	Ohaus Corporation	AR2140

Comments/Description

Made by diluting 10 X PBS (prepared in lab) in distilled water
Needle: BD Precision Glide 300110
Part of the aquactic system

Prepared from solid Tris Base (Promega, H5135), adjusted to pH 7.4-8 with HCl (Merck, 1003171000)

Dissolve 2 or 3% agarose (w/v) in 1x PBS, warm until dissolve.
Dilute 2% (w/v) directly in 1x PBS
Dissolve 4% PFA (w/v) in warm 1x PBS, mix until dissolve in a hot plate in a fume hood. Aliquot and store at -20 °C
Made by diluting 100% ethanol in distilled water
Made by diluting 100% ethanol in distilled water
Made by diluting 100% ethanol in distilled water

C-PLATIN 1 mg/mL. Store at room temperature.
Obtained from local stationary store
Stock solution 20 mg/ml dissolved in water

Set to 37 °C
Model micro15x20

Use 1 mL tips

Dissolve 0.9% NaCl (w/v) in distilled water
Prefer transparent

60 and 100 mm
Obtained from local store
Diluite from stock 20 mg/ml

Obtained from local store

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Editors
JoVE - Journal of Visualized Experiments

February 4, 2020

Dear Editors,

We kindly thank you for the comments and suggestions on our manuscript and video. We went carefully through every point to adjust the changes indicated from the Editorial Board and to respond to the questions raised by the reviewers.

Regarding Editorial and Production comments, I confirm that the manuscript was carefully proofread to correct the spelling and grammatical errors and the references were edited to the correct format. Regarding the video, we edited the jump-cuts and the parts you suggested, and also tried to merge better the procedure with the written manuscript. We also reduced the volume by 3 dB, as asked.

About the reviewer's comments, we will address this question by question.

Reviewer #1:

We kindly thank for your comments on the manuscript and the video. We appreciate your enthusiasm with our work.

Major Concerns:

1. How did the authors arrive at this dosage of cisplatin?
We have added a better explanation in the introduction, as the reviewer suggested. In our lab, we decided the best cisplatin doses on studies of cisplatin-induced AKI of murine models, in which the dose was around 10 mg/Kg (equivalent to 10 µg/g). However, this dose wasn't sufficient to induce kidney damage, so we increased the dose to the ones shown in this study.

2. How is cisplatin stock stored?
In this study, we used cisplatin available in drug stores in our country (C-PLATIN, Blau Farmacêutica). This type of cisplatin is used to treat cancer patients and comes in a dark glass bottle in a dilution of 1 mg/mL in a mixture of sodium chloride, hydrochloric acid, sodium hydroxide, and water (concentrations not specified). The fabricant recommended storing the bottle at room temperature (15-30 °C), protected from light. We added this specification at the beginning of the protocol.

3. It is preferable to euthanize adult zebrafish with an overdose of MS-222. This is well established in the zebrafish field and a veterinary requirement observed by AAALAC/and research university IACUCs.

We appreciate the concern on this field, however, we based our protocols of euthanasia on the Normative Resolution N°37 of the Brazilian Government and the Guide for the Care and Use of Laboratory Animals: Eighth Edition (2011) ("The Guide"), both allows to induce euthanasia by hypothermal shock in tropical species of fishes. This protocol is also recommended and approved by the University of Oregon, where we were trained, and is based on the AVMA Guidelines on Euthanasia and the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (IRAC). Further, we confirmed that the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), mentioned by the reviewer, is based on "The Guide" that reference the same publications that have recommended hypothermal shock as euthanasia method over MS-222 in zebrafish because is less stressful, fast and consistent that the use of MS-222 (Wilson et al. 2009, *Journal of the American Association for Laboratory Animal Science*; Matthews & Varga 2012, *Institute for Laboratory Animal Research*). Because of this, we maintained this part of the protocol as we previously wrote it, although we acknowledge that the approved methods for euthanasia could be different in each country.

4. Rewrite 'with basic functional unit, genetic and developmental similar to mammals'. Suggest: 'and its basic functional unit, the nephron, is conserved with mammals'.
This was corrected in the text.

5. In the video, the researcher is shown monitoring a group of fish after the cisplatin injection. Since the survival rate is high (~30%), it is advisable for the protocol to be edited to suggest that fishes should be singly housed after the injection. This way, fishes are not in tanks with other dead fishes overnight. Furthermore, the protocol should specify how frequently the fishes are monitored (Step 1.9) in the following days. Checking 2 times a day, it would be best practice from a veterinary care perspective (e.g. in the morning and at night, approximately every 12 hours).
We thank for your comments, actually, the fishes were constantly checked the by someone in our team inside the facility where they stayed. The fishes were housed together after the cisplatin injection, fed and monitored for sings of pain or distress, or dead. If any of this happened, the technician would immediately inform to the owner of the experiment. However, we usually did not see dead fishes the first hours after injection and we effectively observed dead fishes only the next day. Nonetheless, we edited this in the video and the text to "monitor the animals twice a day" instead of "next day", as the reviewer indicated.

Minor Concerns: All the minor concerns were related to in-text corrections. We changed all suggestions as the reviewer indicated.

Reviewer #2:

We appreciate your comments and suggestions. This reviewer mentioned a minor concern about the position of the kidney in Figure 1A. We adjusted the position of the kidney and recreated the swim bladder to better understanding.

Reviewer #3:

We kindly thank your comments and suggestions.

Major Concerns:

1. The reviewer suggested a brief introduction of ways we used to assess AKI under stress of any nephrotoxic agent.
We appreciated your comments. We added some information on nephrotoxicity by chemical agents in the introduction to complement this issue.

2. The reviewer also suggests a minor revision of the introduction and the addition of flowcharts to the protocol.
Both suggestions were accepted and added to the manuscript. The flowchart was added as Figure 4.

Minor Concerns:

1. There can be many animals sharing similarities with mammals, why you have specifically selected the zebrafish?
One of the scopes of our lab is to study the regeneration properties of the zebrafish kidneys, for this, we treat them with different probiotics and microbiota-related products and analyze the kidney damage conditions. The available models in zebrafish, at the time that we started this project, were based mainly on the use of gentamycin as a nephrotoxic agent that is also an antibiotic, and this would not be compatible with our treatment (a probiotic bacteria), so we decided to establish a new model using another common nephrotoxic agent, the cisplatin. We added some of this explanation in the text.

2. What are the common routes of exposure of zebrafish to cisplatin under normal conditions?
If we understood well this question, the zebrafish is not exposed to cisplatin in "normal conditions" like in nature. Cisplatin is a controlled antineoplastic chemical that is not available in nature, so zebrafish will not be exposed normally except in a human-controlled manner.

3. Is osmoregulation in this fish common to mammals?
Freshwater fishes like the zebrafish use the kidney like the mammals to regulate osmolarity and water excretion, but also the gills, and in embryonic stages, the skin has also osmoregulatory cells to control ion transportation. It was added a brief explanation in the text.

Thus, we confirm that the manuscript has been read and corrected as the editors and reviewers suggested, as well as we checked that no plagiarism has occurred in the manuscript. We hope you find our manuscript suitable for publication and look forward to hearing from you in due course.

Sincerely,

Camila Morales Fénero
Corresponding Author

NIELS OLSEN SARAIVA CÂMARA'S BIO

Niels Olsen Saraiva Câmara graduated in Medicine from the Federal University of Ceará (1991), has a Master of Sciences in Medicine (Nephrology) from the Federal University of São Paulo (1997) with specialization in Transplantation Immunology at the University of Tours (Diplome des Etudes Appronfodies, France, 1997-1999). He made a PhD in Medicine (Nephrology) by the Federal University of São Paulo (2000) and a postdoc at Imperial College London (2000-2003). His habilitation (Livre Docência) was made at the Federal University of São Paulo (Medicine) in 2006. He is currently full professor of Department of Immunology at the Institute of Biomedical Sciences at the University of São Paulo (since 2011). Visiting Professor at the University of Tours, France in 2006. The laboratory has experience in nephrology area and in cellular and applied immunology, acting on the following topics: kidney transplantation, experimental models of acute and chronic kidney diseases, ischemia and reperfusion injury, regulatory cells and stem cells in kidney diseases. More recently, the laboratory has been studying the microbiota interface, cellular metabolism and inflammation in models of inflammation, transplant, and cell culture. His lab has experience with mouse models and from 2015 he works with zebrafish models of inflammation. Finally, he is a full member of São Paulo Research Academy and full member of the Brazilian Academy of Sciences.

CAMILA MORALES FÉNERO'S BIO

Camila Morales Fénero has graduation in Tecnologia Médica (Biomedicine) with specialization in Histopathology and Citodiagnostic by University of Chile (2012). Her graduation thesis, entitled "Effect of daam1- loss of function on the asymmetric organization of habenular subterritories in zebrafish" was developed in the laboratory of Dr. Alicia Colombo at the Faculty of Medicine in the University of Chile. She made a Master Degree at the University of São Paulo (2015) and developed the project "Effect of short-chain fatty acids (SCFAs) on intestinal inflammation induced by TNBS on zebrafish larvae" in the laboratory of Dr. Niels Olsen Saraiva Câmara. Currently is a senior PhD student at the University of São Paulo working on the effect of microbiota in the inflammation during obesity in zebrafish in the laboratory of Dr. Niels Olsen Saraiva Câmara. She made one year of internship abroad in the laboratory of Dr. Karen Guillemin at the University of Oregon. During her years in Câmara's lab she has supervised four undergraduates and two master students. Has experience in the area of immunology, zebrafish disease models of inflammation, mainly in kidney and intestine, and in the subjects of microbiota, zebrafish germ-free, obesity, intestinal inflammation, acute kidney injury, histopathology, molecular biology and CRISPR-Cas9.

BARBARA NUNES PADOVANI'S BIO

Barbara Nunes Padovani has a Bachelor in Biological Sciences at the University of São Paulo. During her undergrad time (2017-2019) she formed part of the Scientific Initiation Program at Camarâ's lab and developed a study comparing zebrafish wild-type strains during intestinal inflammation. In this same period she participate of the Monitor Program helping to professors in the disciplines Basic Immunology (2018) and Laboratory Methods in Immunology (2019). In 2017 she made an internship abroad at the laboratory of Dr. Carmen Feijoo at the University Andrés Bello in Chile, where she learned some techniques in larval zebrafish such as isolation of intestines in larvae, whole mount immunofluorescence and gavage. Her Completion of Course Work was entitled "Comparison of immune responses and resistance between two zebrafish strains in an experimental model of inflammatory bowel disease" and was developed in the laboratory of Dr. Câmara at the University of São Paulo. She is currently a Master student in the Department of Immunology at the University of São Paulo, working in Câmara's lab in use of *Bacillus subtilis* as treatment for the acute kidney injury induced by cisplatin in zebrafish. She has experience in the area of immunology, intestinal inflammation, microbiota, germ-free derivation, acute kidney injury, molecular biology and CRISPR-Cas9.

MARIANA ABRANTES DO AMARAL'S BIO

Mariana Abrantes do Amaral has a Bachelor in Biological Sciences at the University of São Paulo. During her undergrad time (2018-2019) she formed part of the Scientific Initiation Program at Camarâ's lab studing the effect of short chain fatty acids during intestinal inflammation in zebrafish model and the effect of fructose-enriched diet on regulation of acute renal injury on zebrafish model. In this same period she participate of the Monitor Program helping to professors in the disciplines Basic Immunology (2019) and Laboratory Methods in Immunology (2019). In 2019 she started teaching in Pueri Domus Bilingual School. Her Completion of Course Work was entitled "Short chain fatty acid rescue TNBS-Induced intestinal inflammation in zebrafish larvae" and was developed in the laboratory of Dr. Câmara at the University of São Paulo. She is currently a PhD student in the Nephrology Department at the Federal University of São Paulo, but is supervised and works in Câmara's lab using zebrafish model to study the crosstalk between acute renal injury and liver after fructose induced diet. She has experience in the area of immunology, intestinal inflammation, microbiota, acute kidney injury, molecular biology and CRISPR-Cas9.

GUILHERME JOSÉ BOTTURA DE BARROS'S BIO

Guilherme José Bottura De Barros has graduation in Fundamental Sciences for Health at the University of São Paulo. During his undergrad time she formed part of the Scientific Initiation Program at Câmara's Lab and worked on the role of the platelet activating factor receptor in kidney disease sepsis in mouse model. Then he worked in his Completion of Course Work entitled "Platelets and allograft rejection in experimental skin transplant" in mouse model. He has a Master Degree in Immunology (2019) developed at Câmara's Lab, where he studied the influence of the microbiota in macrophages polarization during acute kidney injury induced by cisplatin in zebrafish. He is currently a Medicine student at University Center São Camilo. He has experience in the area of immunology, acute kidney injury in zebrafish and mouse models, sepsis, and skin transplant.

IZABELLA KARINA XAVIER DE OLIVEIRA'S BIO

Izabella Karina Xavier De Oliveira has graduation in Biological Sciences the University Nove de Julho (UNINOVE). During his undergrad time she formed part of the Scientific Initiation Program at Camarâ's lab and worked on the model of intestinal inflammation in zebrafish. Then she made an internship at Ecolab Quimica making entomologic analysis. She has been working as a technician in Camara's Lab from 2018 attending in the process of maintaining and genotyping mouse strains, making the stocks of solutions, helping in the care and feeding at the zebrafish facility, and helping with experiments in general extracting material, injecting compounds or anesthetizing animals. She has experience doing RNA extraction, genotyping and PCR.

MEIRE IOSHIE HIYANE'S BIO

Meire Ioshie Hiyane was graduated in Biological Sciences from the University of São Paulo in 1996. She has a Master Degree in Microbiology and Immunology from the Federal University of São Paulo (2000) and a Doctorate Degree in Microbiology and Immunology from the Federal University of São Paulo (2007). She worked as Medium Level Technician in the laboratory of Maria Regina do Imperio Lima from 2006 to 2010, working with Trypanosoma cruzi in mouse model. Then she developed works as Superior Level Technician and Lab Manager in Câmara's Lab from 2010 to today. She has experience in immunology, with emphasis on cellular immunology. Acting mainly on the following topics: PCR method with non-palindromic adapters, T cell receptor, Trypanosoma cruzi, trans sialidase, bioplex, mouse maintenance and genotyping and supervisor of lower level technicians.