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Evaluation of zebrafish kidney function using a fluorescent clearance assay.

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Abstract:	The zebrafish embryo offers a tractable model to study organogenesis and model human genetic disease. Despite its relative simplicity, the zebrafish kidney develops and functions in almost the same way as humans. A major difference in the construction of the human kidney is the presence of millions of nephrons compared to the zebrafish that has only two. However, simplifying such a complex system into basic functional units has aided our understanding of how the kidney develops and operates. In zebrafish, the midline located glomerulus is responsible for the initial blood filtration into two pronephric tubules that diverge to run bilaterally down the embryonic axis before fusing to each other at the cloaca. The pronephric tubules are heavily populated by motile cilia that facilitate the movement of filtrate along the segmented tubule, allowing the exchange of various solutes before finally exiting via the cloaca. Many genes responsible for CKD, including those related to ciliogenesis, have been studied in zebrafish. However, a major draw back has been the difficulty in evaluating zebrafish kidney function after genetic manipulation. Traditional assays to measure kidney dysfunction in humans have proved non translational to zebrafish, mainly due to their aquatic environment and small size. For example, it is not physically possible to extract blood from embryonic staged fish for analysis of urea and creatinine content, as they are too small. In addition, zebrafish do not produce enough urine for testing on a simple proteinuria 'dipstick', which is often performed during initial patient examinations. We describe a fluorescent assay that utilizes the optical transparency of the zebrafish to quantitatively monitor the clearance of a fluorescent dye, over time, from the vasculature and out through the kidney, to give a read out of renal function.
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SHORT ABSTRACT:
The zebrafish is a popular tool to model chronic kidney disease (CKD). However, their small size makes it impossible to evaluate renal function using traditional methods. We describe a fluorescent dye kidney clearance assay [1] that allows quantitative analysis of zebrafish kidney function in CKD.

LONG ABSTRACT:
The zebrafish embryo offers a tractable model to study organogenesis and model human genetic disease. Despite its relative simplicity, the zebrafish kidney develops and functions in almost the same way as humans. A major difference in the construction of the human kidney is the presence of millions of nephrons compared to the zebrafish that has only two. However, simplifying such a complex system into basic functional units has aided our understanding of how the kidney develops and operates. In zebrafish, the midline located glomerulus is responsible for the initial blood filtration into two pronephric tubules that diverge to run bilaterally down the embryonic axis before fusing to each other at the cloaca. The pronephric tubules are heavily populated by motile cilia that facilitate the movement of filtrate along the segmented tubule, allowing the exchange of various solutes before finally exiting via the cloaca [2-4]. Many genes responsible for CKD, including those related to ciliogenesis, have been studied in zebrafish [5]. However, a major draw back has been the difficulty in evaluating zebrafish kidney function after genetic manipulation. Traditional assays to measure kidney dysfunction in humans have proved non translational to zebrafish, mainly due to their aquatic environment and small size. For example, it is not physically possible to extract blood from embryonic staged fish for analysis of urea and creatinine content, as they are too small. In addition, zebrafish do not produce enough urine for testing on a simple proteinuria 'dipstick', which is often performed during initial patient examinations. We describe a fluorescent assay that utilizes the optical transparency of the zebrafish to quantitatively monitor the clearance of a fluorescent dye, over time, from the vasculature and out through the kidney, to give a read out of renal function [1,6-9].

INTRODUCTION:
The human kidney plays a crucial role in filtering metabolic waste from the blood and recovering required solutes to sustain cellular homeostasis. There are a number of human genetic diseases that cause kidney dysfunction. The most common inherited renal disease is autosomal dominant polycystic kidney disease (ADPKD) characterized by the development of fluid filled sacs within nephritic tubules; the damage caused by cystogenesis is detrimental to kidney function [10]. ADPKD has an occurrence of 1:800-1:1000 and accounts for 8-10 % of patients in end stage renal failure (ESRF) [11]. Several genes have been implicated to cause ADPKD including polycystin-1 (PKD1) and -2 (PKD2), accounting for approximately 85 % and 15 % of cases respectively [12,13]. Furthermore, the gene products for PKD1 and -2 localize to the cilium and are fundamental to ciliogenesis [14,15]. There is now a recognized family of human genetic disorders, known as the ciliopathies, which affect cilia function and result in CKD [16].

The growing number of human genetic diseases affecting ciliary development and function is drawing global interest in this once considered vestigial organelle. The cilium, a hair-like cellular protrusion, is enriched with receptors and ion channels necessary for the transduction of key cell signaling events. The cilium consists of a microtubule-based axoneme, typically structured into nine radially arranged microtubule doublets with or without a central pair of singlet microtubules. The axonemal structure defines the type and mode of ciliary action. The 9+2 microtubule arrangement confers motility to the cilium where it is utilised in the movement of fluids across epithelial surfaces. The 9+0 configuration is non motile but is believed to mainly

function in cellular signalling events [17]. Apart from CKD, the consequences of ciliary dysfunction are a set of characteristic ciliopathy features that include, obesity, retinal degeneration, polydactyly, and cognitive impairment [16]. However, CKD is amongst the most detrimental to the patient's quality of life and therefore a major driving force behind the development of appropriate *in vivo* models for ciliary related CKD.

The zebrafish is an excellent model to understand the etiology of human genetic disease. Their quick development, production of large number of eggs, transparent tissue, and ex-utero growth allows zebrafish developmental processes to be visualized and biological events manipulated with considerable ease. Genes can be genetically altered using the recent success of genome editing tools (CRISPR [18] and TALENS [19]), knocked down using antisense morpholino technology [20], or pharmacologically regulated by the addition of compounds to their aquatic environment. Indeed, zebrafish offer a platform to undertake experiments that are not permissive in other animal models. Whilst zebrafish are relatively simple vertebrates (compared to humans) they share many functionally conserved organs, genes, and signaling processes in common with humans. For example, the zebrafish kidney is remarkably similar in structure and function compared to humans [21,22]. However, unlike the mammalian kidney that develops through a succession of phases, each marked by a more developed kidney (pronephros, mesonephros, and metanephros), the embryonic zebrafish only develops a pronephros, the most immature form of a kidney. Whilst millions of nephrons can be found forming the building blocks of the mammalian kidney, the zebrafish embryo only possess two. The glomeruli, which allow for the initial blood filtrate, are fused at the midline just ventral to the aorta. Blood filters through the glomeruli into the pronephric tubules that run caudally along the axis, fusing prior to exit via the cloaca. The pronephric tubules are heavily ciliated with motile cilia that are permissive to the flow of filtrate towards the caudal exit [3,4]. This simple pronephric structure maintains zebrafish homeostasis through several weeks of larval growth where they eventually develop into a more complex mesonephros structure [21]. However, the zebrafish never develops a metanephros [21]. Despite the zebrafish idiosyncrasies, the zebrafish nephron is segmented with gene expression profiles equal to that observed in mammals and thus offers an unrivalled *in vivo* model for nephrogenesis [3,22].

Routinely patients are tested for kidney function through a series of blood and urinary tests. Typically the blood is analyzed for dissolved salts, urea and creatinine. High levels of urea, creatinine and abnormal salt concentrations are indicative of problems with kidney function. Urinalysis using a colorimetric dipstick detects abnormal levels of protein, blood, pus, bacteria and sugar present in urine samples. Such tests normally require approximately 30 ml of urine or 5-10 ml of blood. It has been difficult to translate these types of assays to small *in vivo* model organisms, such as the zebrafish, mainly due to the impossible nature of collecting sufficient blood or urine to perform the assay. Here, we address the lack of appropriate zebrafish kidney function tests and describe an innovative technique for its study. By injecting a fluorescent dye into the blood stream we are able to monitor and individually quantify over time the filtration and excretion of fluorescent activity from the blood via the kidney. This method can be used to study kidney damage caused by disease, which we provide an example of.

PROTOCOL:

Ethics Statement: Animal maintenance, husbandry, and procedures are defined and controlled by the Animals (Scientific Procedures) Act 1986. All animal experimentation has been carried out under licenses granted by the Home Secretary (PIL No. 70/7892) in compliance with Biological Services Management Group and the Biological Services Ethical Committee, SGUL, London, UK. All efforts were made to reduce the number of animals used and to refine both procedures and husbandry in order to minimize suffering and enhance welfare.

1. Preparation of Instruments, Anesthetic, and Fluorescent Dye.

1.1) Using a micropipette puller and borosilicate standard wall capillaries (without filaments) pull needles of appropriate length for microinjection (Fig. 1A).

1.2) Make an agarose mould for orientation of embryos for easy injection into the pericardium (Fig. 1B). Glue approximately ten glass microscope slides together to form a 'staircase' of offset slides. For best results, use a drop of rapid set epoxy resin glue in the center of each slide.

1.3) Cut out a section of approximately 78 mm in length halfway through two 10 ml plastic pipettes using a Bunsen burner heated scalpel, remove pipette ends as appropriate. Attach and glue (epoxy resin) pipette sections to the stacked glass slides to provide stability to allow the mould to dip into a 90 mm petri dish. Make every effort to ensure the slide corners align perpendicular to the petri dish floor. Allow glue time to set.

1.4) Cast the mould in a 2 % agarose solution made in fish water (obtained from the aquarium). Pour agarose into a 90 mm petri dish and place the mould on top of the open petri dish, permitting the stacked slide edges to submerge at an angle under the agarose surface. Leave to set on the lab bench for 30 minutes (mins).

1.5) Remove the slide cast and cover the slide-imprinted agarose with fresh fish water containing Tricaine anesthetic (see 1.9) at a 1:25 ratio.

1.6) Make solutions of Rhodamine Dextran (RD) dye. Resuspend Rhodamine B 10,000 MW labeled Dextran in autoclaved ultrapure water to make a stock concentration of 50 mg/ml. For microinjection, further dilute the stock to a final concentration of 5 mg/ml in autoclaved ultrapure water.

NOTE: Pigmented cells begin to differentiate in zebrafish from 24 hours post fertilization (hpf); these can obscure fluorescent markers during image acquisition.

1.7) Inhibit melanocyte formation by using *N*-Phenylthiourea (PTU), which blocks melanogenesis through inhibition of tyrosinase. Make a 0.003 % stock solution of PTU by dissolving PTU powder in aquarium water, heat solution at 60°C to fully solubilize.

1.8) Incubate zebrafish embryos in Methylene blue solution, a mild fungicide, to prevent fungal blooms and increase survival. Add 2 ml of Methylene blue stock solution, containing 0.1 % Methylene blue in ultrapure water, to 1 liter of aquarium water and use as a standard embryo medium.

1.9) Make up a 15 mM stock concentration of Tricaine/Ethyl 3-aminobenzoate methanesulfonate salt as instructed in 'The Zebrafish Book' [23]. Anesthetize the embryos and consequently immobilized them to perform the microinjection procedure.

1.10) After anesthetizing the embryos, orient them, for imaging by using the non-toxic and viscous properties of methylcellulose. To make a 200 ml preparation of 3 % methylcellulose, chill 130 ml of water at -20°C for 30 mins and place on ice. Heat 70 ml of water to 80°C in a glass beaker, add 6 g of methylcellulose and agitate using a glass rod until all particles are wetted and evenly dispersed. Add the ice-cold water, mix, and allow the preparation to cool at 4°C for 30 mins before aliquoting into 50 ml tubes.

NOTE: Lowering the temperature allows the methylcellulose to become soluble. The solution will become thicker as the powder hydrates. 3 % methylcellulose can be stored without bacterial growth for short periods of a week at 4°C or longer at -20°C. Make sure the methylcellulose has reached room temperature before use.

1.11) To perform injections of fluorescent dye into the zebrafish use a standard microinjection set-up (Fig. 1C). This consists of an air compressor connected to a pressure regulator system that feeds into a straight pipette holder for use with 1.0 outer diameter capillaries. The pipette holder should be housed within an MM33 compact 3-axis control micromanipulator secured to a steel base plate by a magnetic stand. Embryos can be visualized, manipulated and injected using a stereo dissecting microscope.

2. Zebrafish husbandry and pre-injection treatment.

2.1) Maintain zebrafish as previously described [23]. Use an aquarium set-up that provides recirculating water supplied at a constant temperature of 28.5°C, conditioned to pH 6.8-7.2 and conductivity of 450-550 µs with sodium bicarbonate and Instant Ocean Sea Salt, respectively. Use wildtype or transgenic zebrafish lines as appropriate to the experiment, maintaining a stocking density of 15 males to 15 females per 8-liter tank. Set the fish facility photocycle to 14 hrs of daylight between 9 am - 11 pm.

2.2) Zebrafish spawning commences at the beginning of the photocycle, when the lights turn on in the morning. To ensure maximum eggs can be collected without disturbing the fish, submerge breeding tanks (containing mesh inserts, available from zebrafish specialists) into wildtype stock tanks the evening before collection.

2.3) On the day of collection, allow 30-40 mins for the fish to spawn, after which collect and rinse eggs using a tea strainer and fresh aquarium water. Deposit the cleaned eggs in a 90 mm

petri dish containing embryo medium and incubate at 28.5°C.

2.4) Treat the embryos with PTU to inhibit melanogenesis. At 8 hpf, transfer viable embryos in minimal liquid to fresh petri dishes containing 1:100 PTU to embryo medium and further incubate at 28.5°C until 72 hpf.

NOTE: PTU can cause developmental defects if used at earlier stages so should be restricted to post 8 hpf stages, but can be treated as late as 24 hpf. Late addition of PTU does not fully block eye pigmentation but is generally successful and inhibiting trunk melanocyte formation.

3. Microinjection.

NOTE: The pericardium incases the heart but is separated for protection and ease of cardiac movement by fluid within the pericardial cavity. The aim of this procedure is to inject RD into the pericardial cavity, this allows for rapid uptake of the dye to the vasculature system.

3.1) Load needles with 6 µl - diluted RD, using microloader tips and secure into the needle holder of the micromanipulator. Break the end of the needle using fine tipped forceps (Fig. 1A). Move the RD solution to the tip of the needle by maximizing pulse duration to the minute setting, switch back to milliseconds once complete.

3.2) Use a stage micrometer, the pressure regulator, and refinements to needle tip length (using forceps) to adjust the size of the expelled droplet to 100 µm in diameter, this equates to a 0.5 nl volume (Fig. 2A).

NOTE: When breaking the needle, be careful not to break too much off otherwise it will make calibrating the injection volume difficult. If necessary, further break the needle tip to increase the drop size however, maintain a needle thickness that permits entry into the pericardium without excessive bending or damage to the tissue.

3.3) Prior to injection, anesthetize the embryos in embryo medium containing Tricaine. Set-up two 35 mm petri dishes containing 5 ml embryo medium, label one 'Tricaine' and the other 'Recovery'.

3.4) Add 200 µl of stock Tricaine to the appropriately labeled dish. Once ready to inject, select an individual embryo at 72 hpf and transfer in minimal liquid to the Tricaine dish. Monitor the activity of the embryo, test the embryo is anesthetized and immobilized by gentle agitation using a truncated microloader tip.

3.5) Transfer the anesthetized embryo to the injection mould and orientate the embryo within an agarose trough so the left side is facing up, positioning the heart to the left of the field of view.

3.6) To inject the RD into the heart, pierce the pericardium with the needle and inject 1 nl of

RD into the pericardial cavity of the anaesthetized embryo (Fig. 2B, right panel). Withdraw the needle after injection. Transfer the injected embryo in minimal liquid to the 'Recovery dish' and monitor for 1 min. Transfer the individual embryo to a 24-well plate containing 1 ml fresh embryo medium (containing PTU) and label appropriately.

NOTE: The pericardium is tough, however there is a notable weak point at the intersection where the pericardium meets the ventro-caudal pharyngeal arches and dorso-rostral yolk sac (Fig. 2B arrowhead). The needle should be directed to this groove. When the needle is in place, a firm tap of a finger on top of the micromanipulator will facilitate entry into the pericardial cavity.

3.7) Repeat steps 3.2 - 3.6 for at least 10 embryos per experimental group, place each fish in a separate well and uniquely label to permit individual experimental follow-ups. Incubate at 28.5°C for three hours.

4. Imaging Acquisition

4.1) At 3 hours post injection (hpi) anesthetize embryos as previously described and transfer to a 35 mm petri dish containing 3 % methylcellulose. Gently push embryos into the methylcellulose and orientate so the lateral side can be imaged.

4.2) Acquire an image of the embryo under UV, using a filter for visualization of emitted light at 570 nm (Fig. 2C). After image acquisition, allow the embryo to recover before replacing in the designated well. Acquire images for all injected embryos and further incubate at 28.5°C.

NOTE: In this protocol we used a fluorescent stereomicroscope with a TXR filter set, a DFC300FX camera and respective application software. Make a note of the exact acquisition settings for subsequent image acquisition.

4.3) At 24 hpi, acquire a second round of images as described in section 4.1. Once all the images have been acquired, at both 3 hpi and 24 hpi, humanely dispose of the embryos or use for other experimental means e.g. immunohistochemistry.

5. Image processing

5.1) Quantify fluorescent intensity of each injected embryo, at 3 hpi and 24 hpi, by analyzing images using NIH's ImageJ software. To measure fluorescent intensity, open an image in ImageJ and specify a region of interest (roi) at 100 px^2 (Edit<selection<specify).

5.2) Position the heart in the center of the roi (Fig. 2C), set measurements to include mean gray scale and area (Analyze<set measurements), perform measurement (Analyze<measure). Complete for each set of images, at 3 hpi and 24 hpi, per embryo and transfer average gray scale values to a spreadsheet for further processing.

NOTE: We selected a fixed roi size of 100 px^2 as this encompasses individual hearts between embryos. The heart was selected to perform measurements due to its large size that enables a convenient location to measure fluorescent content of the blood prior to entering the kidney.

5.3) Perform statistical analysis using appropriate statistical software. Compare groups for statistical significance using a student's t-test.

REPRESENTATIVE RESULTS:

Bardet-Biedl syndrome (BBS) is a rare heterogeneous ciliopathy that affects approximately 1:160,000 people worldwide [16]. Patients present with a number of associated problems including polycystic kidneys, subsequently patients frequently require kidney dialysis or transplantation [24]. ESRF is the most common cause of death in BBS, with around 30 % of patients developing CKD [16]. Currently, 20 non-related genes have been implicated in BBS with no published genotype-phenotype association. The BBS proteins share common protein localization domains within the cilium and basal bodies that, along with the patients' characteristic traits, infer a ciliopathy diagnosis. BBS9 encodes Parathyoid Hormone-responsive B1 (PTHB1) protein that together with other BBS (BBS1, BBS2, BBS4, BBS5, BBS7, BBS8) proteins form the core BBSome complex responsible for the formation of the primary cilium [24]. Mutations in BBS9 account for 6% of BBS cases [24]. Furthermore, PTH has been implicated in kidney cyst formation through the promotion of renal epithelial cell proliferation, suggesting that loss of *bbs9* function in zebrafish might display renal defects [25]. Previous reports using a *bbs9* knockdown zebrafish model exclude a description of the kidney, presenting the opportunity to demonstrate the described kidney function assay [26]. Knockdown of *bbs9* function was achieved in zebrafish by injecting, at the 1-to 4- cell stage, an antisense morpholino to block gene specific *bbs9* translation and in parallel a standard negative control morpholino against an intronic mutation in human beta-globin (4 ng *bbs9*MO, sequence: GGCCTTAAACAAAGACATCCTGTAA and 4 ng control MO, sequence: CCTCTTACCTCAGTTACAATTTATA). We found that loss of *bbs9* function resulted in 40% of embryos displaying pronephric cysts by 5 dpf, suggesting this was an appropriate model to analyze kidney function using the Rhodamine dextran clearance assay. We observed that morphant fish have a significant reduction in their ability to clear the fluorescent dye after 24 hpi compared to controls (Fig. 3) con: 14.8 SEM ± 1.2, n=9; *bbs9*MO: 61.0 SEM ± 10.3, n=10; unpaired t-test P value: 0.002). To rule out the potential that reduced clearance could be due to reduced blood circulation, embryos were evaluated for heart beat and recirculating blood cells, both control and morphant fish had comparable heart rate and blood flow. These data indicate that kidney function is impaired in *bbs9* morphants. Indeed, *bbs9* morphant embryos develop cystic pronephric tubules concurrent with that observed in BBS patients.

FIGURE LEGENDS:

Figure 1: Equipment preparation and set-up.

A) Borosilicate capillaries should be pulled for microinjection using an appropriate needle puller. The needle requires breaking with forceps (dashed line) to permit the RD to exit the needle, care should be taken not to break the needle too much rendering a needle that cannot be calibrated. Scale bar: 200 µm. **B)** An agarose mould for embryo manipulation and orientation can be

fashioned by gluing together glass slides and plastic pipettes. **C)** The standard microinjection set-up.

Figure 2: Microinjection of Rhodamine dextran into the pericardial sac.
A) Calibrate the drop size to 10 increments (100 µm) on a 1 mm stage reticule. **B)** Correct positioning of the needle (black arrows) into the crevasse marking the intersection between the caudal pharyngeal arch, yolk sac and heart will facilitate piercing the tissue (arrowhead). Scale bar: 200 µm. **C)** Rhodamine dextran can be seen rapidly taken up by the whole vasculature (white arrows). 100 pixel ^{2} regions of interests covering a centralized heart (yellow square) should be used to take measurements of the mean gray value and hence pixel/fluorescent intensity.

Figure 3: Knockdown of *bbs9* causes defective kidney function in zebrafish.
A) Embryos injected into the pericardium with Rhodamine dextran at 3 hpi and 24 hpi, top and bottom two panels respectively, in either control or *bbs9* morphant embryos. Arrowheads indicate the heart; arrow indicates the formation of a pronephric cyst. **B)** Percentage fluorescent intensity remaining after 24 hpi in control versus *bbs9*MO embryos. Error bars show the standard error of the mean (SEM), ** P≤0.01.

DISCUSSION:
Zebrafish offer a valuable tool to model human genetic disease, their use as a scientific instrument for *in vivo* research have enabled detailed studies of the genetic breakdown of many biological systems, including the kidney. Much is now understood about how the zebrafish kidney develops and functions. The striking similarities to human nephrogenesis and homology with disease causing genes [21] has illustrated how zebrafish have become fundamental in understanding how defects in gene function lead to the pathology of renal disease. Indeed, genetic manipulation can be effortlessly achieved in zebrafish using antisense morpholino knockdown technology or more advanced targeted genome editing tools such as TALENS or CRISPR. Creating knockdown or mutant models for suspected renal disease causing genes is the first step in understanding their involvement in the disease manifestation. To do this a reliable assay for kidney function is sought to indicate whether a candidate gene is likely responsible for the pathology observed in patients.

Kidney function tests are straightforward and relatively cheap to perform on humans, generally looking at levels of solutes present in the blood or urine. However, these methods are inapplicable in zebrafish due to its small size and aquatic habitat. The Rhodamine dextran assay described here utilizes the ability of pronephric tubules to filter low molecular weight components from the blood. The podocytes positioned within the bowman's capsule of the kidney, that envelopes the capillaries of the glomerulus, allow the free passing of small molecules such as water, ionic salts and glucose through a slit diaphragm [27]. The filtration slits further function to prevent the loss of macro proteins from the blood. Filtration is restricted for molecules above 5 kDa and almost completely blocked at the size of serum albumin [28] (approximately 65 kDa). By injecting a fluorescent dextran dye of approximately 10 kDa, at a known concentration into the pericardial cavity, we are able to measure fluorescent intensity of

the blood over time. Under normal conditions approximately 85 % of initial fluorescence is lost from the blood, over a 24-hour period, through secretion via the kidney. One should note that the injection into pericardial space is only possible with low MW dextran that passes freely into the vasculature. Thus, this assay only gives a read-out for the rate of clearance for low molecular weight components and does not give any information about the efficacy of glomerular filtration. The latter can be assessed more directly using high molecular weight dyes over 70 kDa, requiring injection into the vasculature and not into pericardial space. Indeed, dextrans of various MWs can be used to further dissect kidney function [28].

The zebrafish pronephric tubules are highly ciliated with motile cilia that facilitate the movement of filtrate toward the cloaca [3,4]. Defects in ciliary machinery have been implicated in kidney disease. BBS is a genetically heterogeneous, autosomal recessive disorder characterized by childhood-onset retinal degeneration, early onset obesity, cognitive impairment, polydactyly and renal malformation [24]. To date, twenty *BBS* genes (*BBS1-20*) have been identified. Disruption of *bbs* leads to renal cyst formation and defective pronephric function in zebrafish [9]. BBS9 interacts with other BBS proteins to form the BBSome responsible for appropriate ciliogenesis, mutations of which account for 6% of BBS cases [24]. Whilst a zebrafish *bbs9* knockdown model has been reported, a description of the renal phenotype was lacking [26]. By knocking down *bbs9* in zebrafish, using the morpholino approach, we demonstrate the use of the renal clearance assay as a method to determine kidney function in a kidney disease model. Here, we show that *bbs9* morphant embryos display impeded fluorescent clearance from the blood stream, indicating that the kidney failed to remove low MW solutes. Thus, because of the involvement of *bbs9* in ciliogenesis, and the role of pronephric cilia in facilitating filtrate movement, the observed kidney clearance in morphants is likely to be due to aberrant cilia function. This method represents a valuable tool for assessing kidney function in zebrafish disease models.

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DISCLOSURES:
The authors have nothing to disclose

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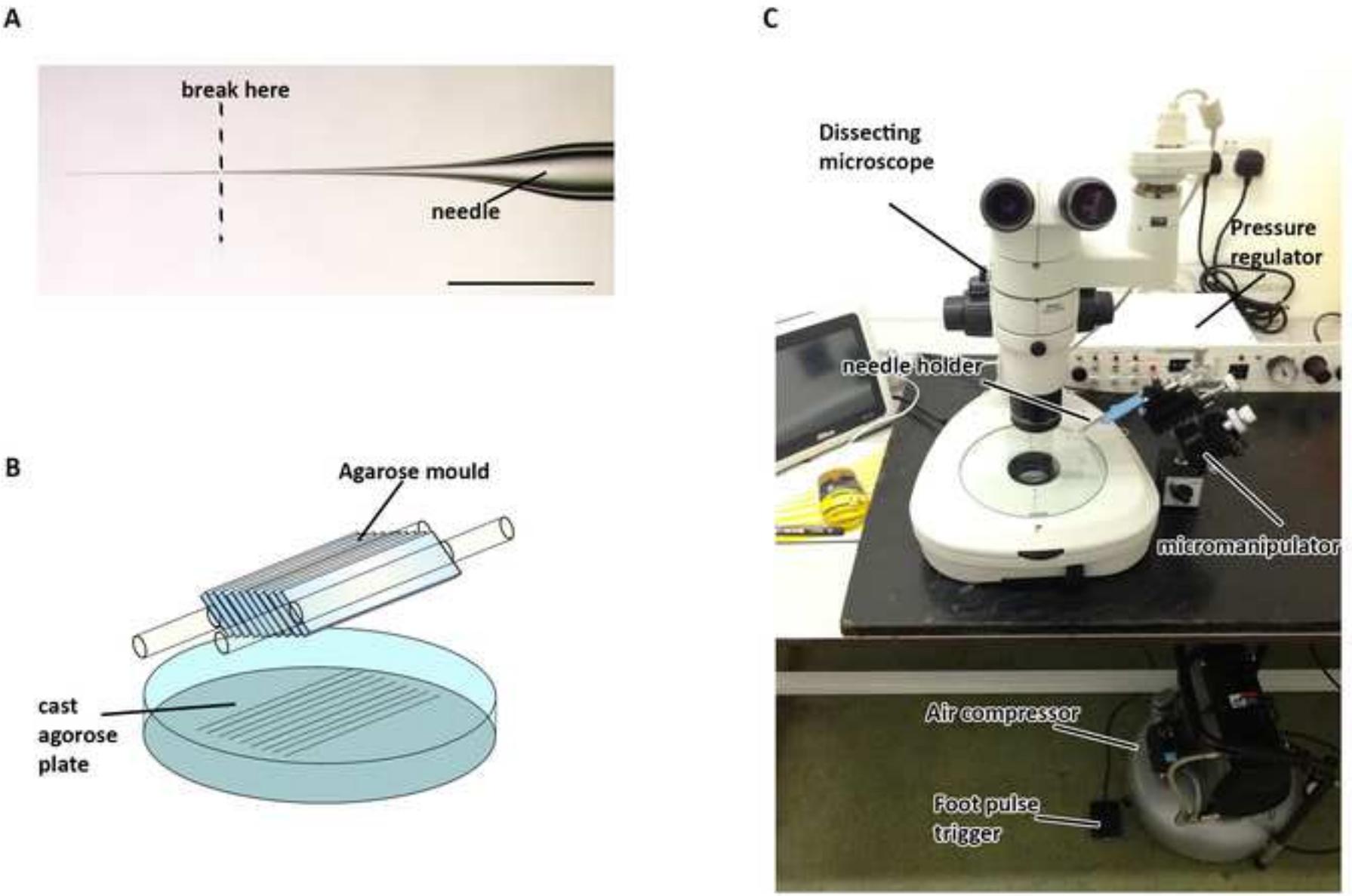

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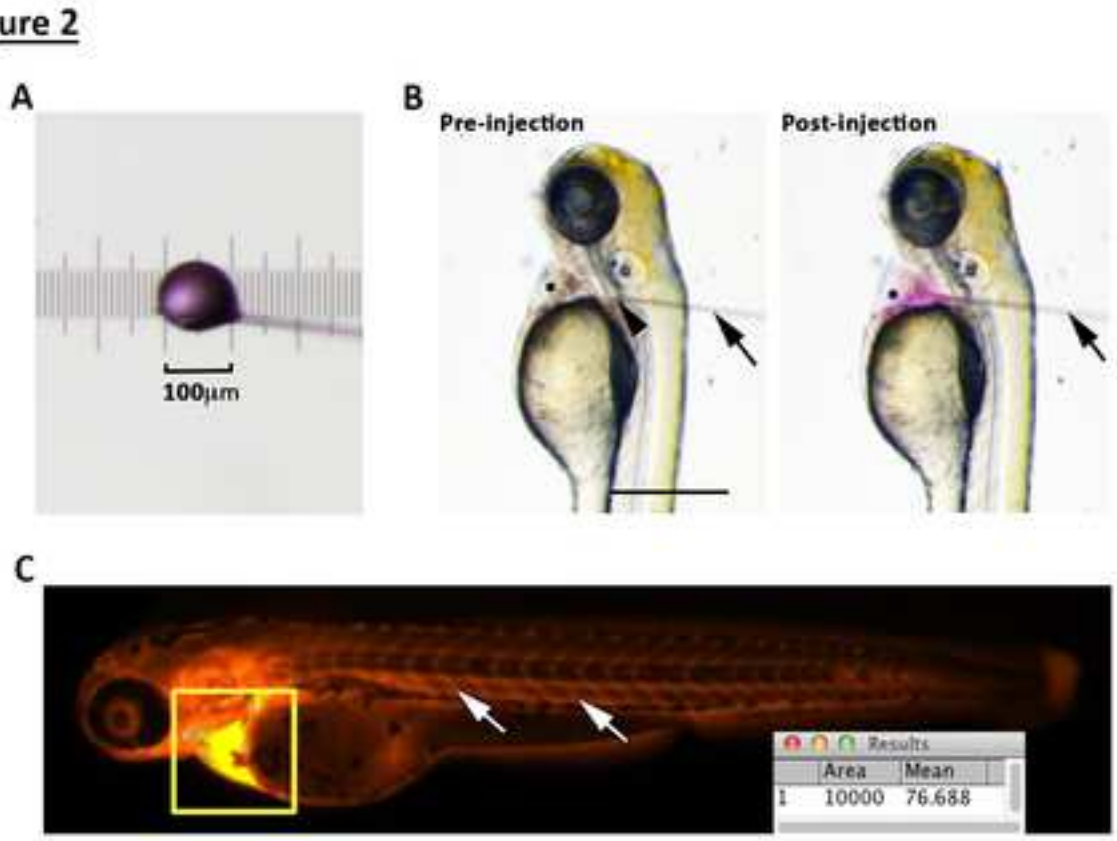

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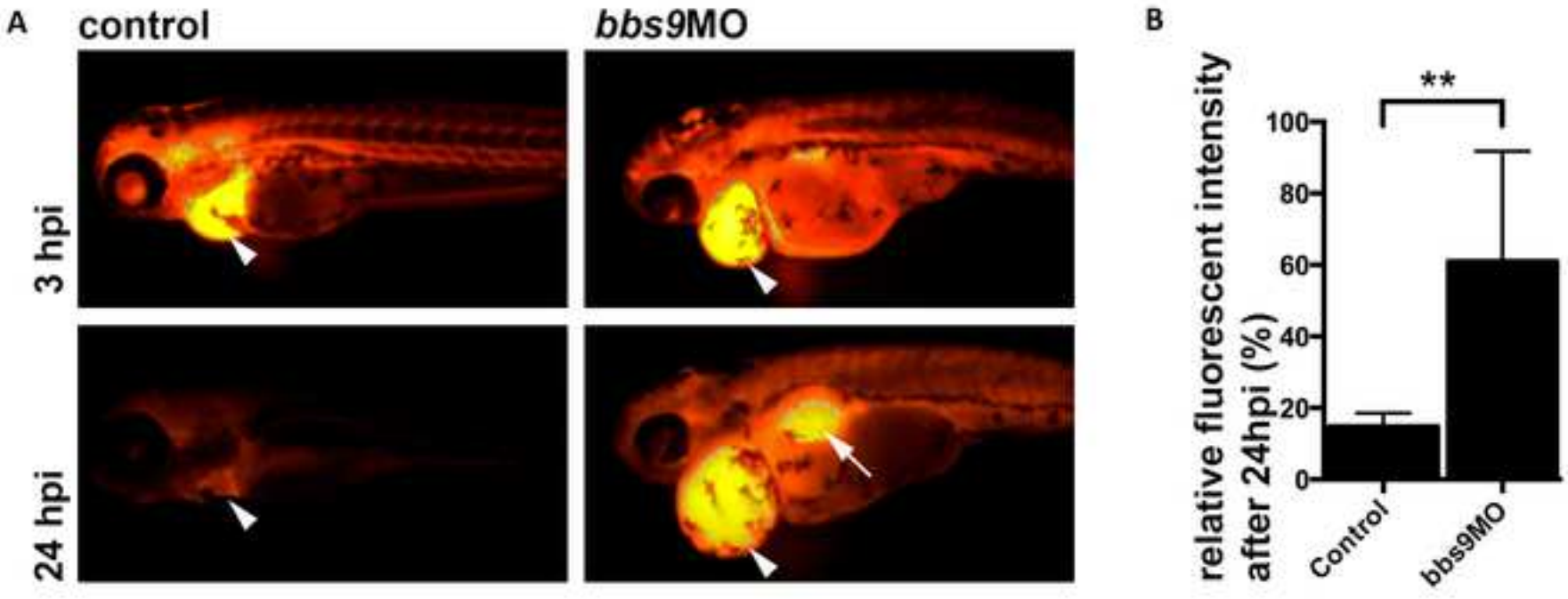

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Dear Dr Upponi,

Re: Revision of manuscript entitled "Evaluation of zebrafish kidney function using a fluorescent clearance assay"- JoVE52540R1.

We have carefully considered the reviewers comments and have prepared a point-by-point document that addresses each comment in turn. In summary, we have successfully completed all the textual corrections and minor comments suggested by the reviewers. In addition, we have addressed many of their major concerns that we outline in the attached document.

Thank you for the valuable reviewers comments, I look forward to your reply.

With best wishes

Daniel Osborn

9th September 2014

Reviewer 1:

Major concerns:

Justification of why the authors have chosen to target zebrafish bbs9 specifically and how their MO compares to previous published bbs9 MOs needs to be improved. The MO presented in this report gives a severe kidney phenotype, as observed by the reduced clearance of RD and the development of renal cysts, the latter being a phenotype that has not been described with two previously published bbs9 MOs (Veleri et al., 2012, PLoS One, 7, e34389).

We have now included additional information explaining the rationale behind using bbs9 knockdown to demonstrate the rhodamine dextran assay. Firstly, Bbs9 encodes parathyroid hormone responsive B1 (PTHB1), where PTH has been implicated in renal cyst formation through the promotion of kidney epithelial cell proliferation. Secondly, Bbs9 has been shown to interact with other bbs proteins to form the core BBSome responsible for the formation of the primary cilium. Mutations in all 20 BBS genes, including BBS9 (representing 6% of BBS cases), have been shown to cause renal defects with no genotype-phenotype correlation. These points suggest that Bbs9 is likely to be a good candidate that when knocked down might display a kidney phenotype in zebrafish. We suggest that previous publication failed to document or comment on a renal phenotype because they mainly focus on the brain and eye phenotypes at stages where the renal cyst have yet to form. Pronephric cysts in zebrafish become apparent from 72 hpf onwards. At 72 hpf the cysts can only be convincingly shown by sections through the pronephric tubules to identify dilated kidney lumens. At 4 dpf (the stages we analyse), cysts can be observed down the microscope without the need to section embryos. Furthermore, we report cyst formation in 40% of embryos indicating that the phenotype does not have 100% penetrance so may have been missed in previous reports.

To show that this assay can also be informative for less severe phenotypes, a dose-response curve of the bbs9 MO should be performed. What are the limitations of the assay in subtler phenotypes?

This is a great suggestion from reviewer 1, however we feel this experiment is not necessary for a technical paper such as JoVE. Our remit was simply to demonstrate the use of the rhodamine dextran clearance assay to identify defects in kidney function in zebrafish. Here, I feel we go above and beyond to satisfy this.

It would also benefit the reader to see that rescue of the phenotype with human BBS9 mRNA restores renal function using their assay, validating this assay for functional testing of allelic variants of unknown significance found in patients.

Again, a great experimental suggestion. However, it is in our opinion that these experiments should be confined to more research based articles.

It is unclear why the authors have chosen for a 100 pixel2 around the heart to analyze fluorescence intensity. Could the same results be achieved when measuring fluorescence in the entire embryo?

Measurements of the heart avoids the autofluorescence associated with yolk cells, which might obscure the result if the whole embryo was used. Furthermore, we wanted to measure fluorescent content at a point in the system before the kidney, before filtration. We have added a note in our image processing section that clarifies this point: “We selected a fixed roi size of 100 px^2 as this encompasses individual hearts between embryos. The heart was selected to perform measurements due to its large size that enables a convenient location to measure fluorescent content of the blood prior to entering the kidney”

The authors measure mean gray values of the 100 pixel2 region, however should also normalize for background autofluorescence that could influence results, specifically for less penetrant phenotypes.

Thank you for this suggestion. However, we feel this is an unnecessary step due to image acquisition parameters remaining constant between embryos and timepoints. This means that any autofluorescence in the heart will be accounted for and normalized between experimental embryos and controls. Furthermore, since each embryo is tracked independently any reduction is specific to that particular embryo where autofluorescence would remain consistent.

It is unclear what the authors mean by 'control' to which they compare their bbs9 MO results. Are these embryos injected with scrambled MOs (preferred control) or a sham injection, or uninjected at single cell stage? Comparing the renal clearance in either sham-injected or uninjected animals is not an adequate control.

Apologies for not including this in the original manuscript. Our control is a standard negative control morpholino, against an intronic mutation in human beta globin, injected in parallel at the same concentration as the *bbs9* morpholino. This is now included in the text.

Reviewer 2:
Major concerns:

In the author's abstract and introduction, the description of pronephros anatomy is misleading. The pronephric kidney in the zebrafish is composed of two segmented nephron tubules, but the authors refer to these repeatedly as pronephric ducts (PND). For example, in the abstract (lines 49-50) the authors refer to the zebrafish kidney nephrons as "pronephric ducts". In the introduction (lines 109-110) the authors use the terms pronephric tubules stating, "Blood filters through the glomeruli into the pronephric tubules and into the pronephric ducts

(PND). As Wingert et al., 2007 have described, the PND is just one distal segment of each pronephric tubule. Furthermore, the authors state in lines 111-112 that the PND are heavily ciliated, it is known that the proximal segment regions of the pronephric tubule also contains multiciliated cells (Ma and Jiang, 2006; Liu et al., 2006; Li et al., 2013). I think a bit more scrutiny on this subject is needed and furthermore that the aforementioned references should be included.

Thank you to Reviewer 2 for pointing out this discrepancy. We have clarified these points in the main body of text and have avoided using the term pronephric duct by describing the pronephric tubules as a whole, we believe this avoids any confusion.