

Justin Cherny, Ph.D.

Science Editor

JoVE

Editorial Office

17 Sellers St.

Cambridge, MA 02139

December 26, 2013

Dear Dr. Cherny,

I am pleased to submit our revised manuscript **JoVE 51644 (Revision 2)**, entitled “**Analysis of Nephron Composition and Function in the Adult Zebrafish Kidney.**” We believe the current version is vastly improved and will provide the community with a very valuable guide to nephron studies in zebrafish.

We edited the manuscript based on the helpful recommendations from the panel of reviewers. One common question they had regarded the specificity of the labels for the various segments of adult kidney nephrons: namely, if/how each label related to other gene expression domains and whether particular labels overlap or indeed mark distinct subsets of cells. We realized a major weakness in the manuscript was the omission of dual labeling data for our techniques and side-by-side comparisons to whole mount *in situ* hybridization (WISH) data. While we were confident in our anatomical assignations due to our extensive research on nephrons, the reviewer comments helped us to appreciate that we needed to SHOW readers many more representative results and provide dual labeling technique instructions. **Thus, we have sought to document how our nephron labels are compatible in various combinations, and incorporated these multi-step staining procedures into the revised manuscript.**

**We now provide readers with a bounty of images that fully demonstrate each nephron segment assignation using double, and in some cases triple, labeling procedures.** As a result, you will see that each figure has been elaborated with many additional images and labeled in exquisite detail. This set of figures substantiates our findings and reinforces data from our prior publications on this topic (Wingert, *et al., PLoS Genet*, 2007; Wingert and Davidson, *Dev Dyn*, 2011; Diep, *et al., Nature* 2011; Gerlach, Schrader, Wingert, *J Vis Exp*, 2011; Li, *et al., Dev Biol*, 2013 in press). **While these papers** (and those from colleagues, e.g. Zhou and Hildenbrandt*, Am J Physiol Renal Physiol*, 2010) **were cited in the previous version, some crucial WISH adult nephron data were originally published in the lengthy Supplemental Figures for the Diep, *et al.*, 2011 study**, and indeed were part of my own contribution to that study. *Due to the location of WISH data in supplemental figures, we suspect that the community is poorly acquainted with these data*—which would naturally explain the reviewer questions about how nephron labels compare to known gene expression domains. We have incorporated single and double WISH data into Figures 4, 5 and 8 so that *JoVE* readers have all the information annotated together in this paper. **This now makes our methods paper the most current and comprehensive resource compiled to date concerning the molecular characteristics of the adult zebrafish kidney.**

**Several other major revisions were performed.** To help familiarize readers with nephron segment labels and help them to understand the relationships between different labels, we created a table of nephron segment characteristics (now in Figure 1) to summarize our representative results. We also constructed a flow chart of methodologies (now Figure 2) to map how the labeling procedures in the video article relate to one another. Since we show dual nephron labeling from cryosections, we have added cryosectioning and immunohistochemistry staining to the protocol. Since we added WISH data, we have included a guide for the different steps that we use to process WISH samples in conjunction with our standard WISH protocol (Cheng, *et al., JoVE,* 2013 in press). As Cheng, *et al.* will be published as a *JoVE* open access article, there will be no restriction for readers of this kidney methods paper to gain easy access to the full WISH protocol employed by our lab.

**Finally, we have addressed each of the minor concerns raised by the reviewers.** We have edited parts of the introduction, results, and discussion to clarify these items. On the following pages is the point-by-point response.

We wish to thank both you and the reviewers for taking the time to provide considerate and useful suggestions that have enabled us to better evaluate the original version and now craft a dramatically more detailed, and thus we believe improved, methods manuscript on this topic.

Sincerely,



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***JoVE 51708 R1: Response to Reviewers' comments***

***Reviewers' comments:***

***Editorial comments:***

*1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.*

This is the file we used for subsequent revisions.

*2) Please disregard the comment below if all of your figures are original.*

*If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

Our figures are entirely original.

*3) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.*

We have proofread for spelling and grammar issues.

*4) Please ensure steps 3.3/5.5 refer to the correct steps.*

We have checked that steps 3.3 and 5.5 are referring to the correct prior steps in the protocol.

***Reviewer #1:***

*Manuscript Summary:*

*Authors describe new additional methods to visualize the adult zebrafish kidney nephron to evaluate renal reabsorption and to assess nephron tubule composition. Some techniques are not well described or incomplete for users who will read this manuscript. Although their new methods is highly accepted and can share with the kidney community.*

We thank Reviewer 1 for their positive review of the manuscript, including the feedback that these methods are highly accepted in the nephrology field and will be useful to share with the zebrafish community in this methods article format. The comments suggesting how to clarify several steps in the procedures were very helpful. We have addressed each point below, and each alteration in the revised manuscript is highlighted in grey.

*Major Concerns:*

*1. (page 5) Why authors chose only 5-7 months old zebrafish? Any reason behind?*

Yes, we have the best results with kidney samples of this age range. Younger fish can have small kidneys that are difficult to dissect, and kidney samples from old fish can contain masses of scar tissue in the organ that cannot be analyzed. We have added this information to the Note located in Step 1.3.

*2.Does author need to use 20 micro-liter for injection volume to use for ip injection? Other zebrafish user has been using smaller volume. 2 micro-liter can be good enough, but they tried and they need ten time more? Better to describe body weight for zebrafish that people can compare to the mice model ip injection. If you think about the volume for ip inject used for mice, is it appropriate to use this volume for small adult zebrafish?*

Yes, we use a 20 microliter volume for injection. This has no adverse side effects—the volume literally corresponds to two drops of fluid. This volume corresponds with several published protocols for the injection of fluid (dextran or injury agents like gentamicin) into the adult zebrafish abdominal cavity (Diep, *et al.*, 2011; Drummond and Davidson, 2010). Body weight for zebrafish has not been adopted as a standard measure in the field (Diep, *et al.*, 2011; Drummond and Davidson, 2010).

*3. Water is used for diluting dextran stock for use? What diluted concentration they are using or are they using stock solution to inject? Explanation is missing.*

Yes, we used distilled water for diluting dextran powder (this recipe is in Step 1.1). We also added this information to the table of materials for easy reference. Adult fish can be injected with the stock concentration of 50 mg/ml. Diluted stocks also work, and can reduce background fluorescence. We indicate the dextran dilution that is still effective at specific PCT segment labeling in Step 1.5.

*4. Authors describe that, "Uptake of dextran by the proximal tubule segment occurs within 8-12 hours and can be detected typically for at least up to 3 days post-injection." The data they showed for Fig. 1-5, is when?*

These data are now located in Figures 3 and 4. In both instances, the data shown is 3 days post dextran injection. This detail has been added to the corresponding Figure legends.

*5. In 2.8, 3.11 sections kidney needs to be placed in glass tube for particular reason, or they can place in eppendorf tube?*

The use of a glass vial for handling of adult kidney samples facilitates visualization of the tissue and allows for washes with large volumes (relative to the size of the tissue) of approximately 5 ml. We edited the notes in Step 2.8 and Step 3.5 to indicate the rationale for using a glass vial. Alternatively, a 12-well cell culture dish can be used. While a plastic microcentrifuge tube could be used, the standard sized tubes (1.5 ml) could restrict the washing.

*6. In 3.12, 0.05% PBS-Tween means exactly what? PBS with 0.005% Tween or something else?*

This means 1X Pbs with 0.05 % Tween, not the more commonly used 1X Pbs with 0.1 % Tween. We have clarified this point in the methods steps and added the solution recipe to the materials table.

*7. Entire protocol lack volume of the solution they used for some cases of their description. Some is described to use glass tube but some lack information. Glass tube need to be used instead of eppendorf tube is unclear.*

As noted in our response to point #5 above, the use of a glass vial for handling of adult kidney samples facilitates visualization of the tissue and allows for washes with large volumes. Throughout the steps we have carefully noted volumes for other wash steps to provide this missing information.

*8. They also lack information, what filters they used to take images for the figures they used for the manuscript.*

A standard Hoechst/DAPI filter was used for DAPI, dextran-cascade blue and alkaline phosphatase detection. A standard FITC filter was used for dextran-FITC, dextran lucifer yellow, and anti-GFP detection. Standard TRITC or Texas red filters were used for dextran-fluoro-ruby, propidium iodide, and DBA. The catalog numbers for the exact filters we used with our Nikon stereomicroscope and compound microscope have been added to the table of materials.

*9. Authors do not describe particular comments of dextran size for the use of zebrafish. They should include the information.*

The specific dextran item numbers were provided in the materials table, and each corresponds to particular sized dextran moieties. We have used both 10 and 40 kDa size dextrans to label the proximal tubule cells via endocytosis of the label. To provide readers with details in the paper, so that they would not have to look the item number up in the manufacturer’s listing online, we have added the molecular weight of each dextran to the materials table and edited the legends of Figures 3 and 4 to also specify the molecular weights.

*10. Author describes that "Alkaline phosphatase labeling also marks the proximal tubule, with strong reactivity in the PCT and slightly weaker reactivity in the PST. Finally, the DBA staining enables labeling of distal tubule segments, which display a characteristic branched morphology." They do not explain using their figures.*

We added labels to indicate these regions in the figures, and describe this in the representative results section.

*11. Author describes that, 2.11) Place small pieces of modeling clay on each corner of an 18 x 18 mm glass coverslip and slowly set the coverslip onto the kidney. Explanation needs more clarification. What is modeling clay and how much thickness or space need to be made by clay?*

To clarify, we provided an image in Figure 1, with a metric ruler positioned alongside the prepared kidney slide preparation for easy reference and reader comprehension.

***Reviewer #2:***

*The manuscript by McCampbell and colleagues describes a protocol of how to label different tubular parts of the adult zebrafish kidney, the so-called mesonephros. I believe these labeling techniques to be very useful for researchers in the field of kidney regeneration and function.*

We thank Reviewer 2 for their comments and feedback, along with the compliment that the methods will be very useful for researchers in the field of kidney regeneration and function. The suggestions they made have been very helpful in revising the manuscript. Edits in the revised manuscript are highlighted in grey.

*There are some points that should be considered or improved:*

*1) The introduction is inappropriately long and should be reduced by at least 50%. While interesting all the "human" aspects can be deleted, at least dramatically condensed.*

The introduction abides by the standard formatting length for *JoVE* papers. We have utilized a majority of the allowed space (which is 1500 words) in order to provide a useful resource for the zebrafish community, in particular to provide a context in which to consider the use of zebrafish for kidney research. Explaining the state of knowledge about kidney regeneration is vital, as that provides the backdrop for both why and how zebrafish can be used to address deficiencies in our knowledge. The suitability of the introduction was actually complimented by Reviewer 4 and was not a point of concern raised by the other reviewers. We have, however, partly condensed the coverage on human kidney topics in order to make space to add more details about nephron structure, as suggested by Reviewer #3 (major concern point 1).

*2) Protocol: 3.11; can you indicate how long depigmentation takes?*

We revised Step 3.11 to indicate that depigmentation typically takes 20 minutes but occasionally can take longer.

*3) Figure 1: Are two different magnifications really required? B/C or D/E would be sufficient.*

We have kept the images that were originally located in panels B/C and eliminated the images in D/E to make space for additional images (note Figure 1 is now Figure 3).

*4) Figure 3: Are four different magnifications required? I think two would be fine.*

We have kept two images and eliminated the additional magnifications (note Figure 3 is now Figure 4).

*5) Figure 4: In the text describing the figure, expression of markers slc20a1a, trpm7 and slc13a1 are mentioned. However, there are no data shown. It therefore does not become clear or convincing that the particular segments labeled by fluoro-ruby are indeed the ones identified by the respective markers. Either the marker expression has to be shown (by ISH or IHC) or the hints regarding the markers should be deleted. Also, in at least one of the panels PCT and PST should be indicated.*

To address this concern, we have added images to the aforementioned Figure (note this is now Figure 5) of whole mount *in situ* hybridization gene expression patterns for several proximal transcripts, including a pan-proximal marker (*cubilin*) that matches the pattern of alkaline phosphatase labeling, the PCT markers (*slc20a1a, mafba*), and a PST marker (*slc13a1*). Further, double *in situ* hybridizations are provided of *slc20a1a* and *slc13a1*, demonstrating that the segmental expression domains of these markers are directly adjacent and do not overlap. Markers for distal segments are shown in Figure 8.

It should be noted that the markers we mentioned have been previously published (e.g. Diep, *et al.*, 2011; Gerlach, Schrader, Wingert, 2011), and we provided these citations in the original manuscript. The images we provide here represent further validation of the segmental domains and are provided ultimately to facilitate easy comparison of the domains for the *JoVE* readership.

*6) Figure 6: the major collecting duct is demarcated by a white dotted line. What is this based on? Another visualization of the collecting duct should be shown.*

The white line was drawn based on a brightfield image, which unfortunately was not saved in our files. We have not identified a marker that is specific for the major collecting ducts, which are distinctive based on their very wide diameter and location along the anterior-posterior axis of the kidney (See Figures 6 and 7 for clear examples), which can be faintly visualized due to background levels of fluorescence when kidneys are treated with alkaline phosphatase. The major collecting ducts have been labeled in these Figures.

***Editorial comment:***

*[Please keep JoVE's protocol guidelines and length requirements in mind while addressing reviewer comments (use short steps, imperative tense, proper spacing, etc).]*

Yes, we have done so.

***Reviewer #3:***

*The manuscript by McCampBell et al. describes methods to visualize different nephron segments in the zebrafish kidney. It is a very important resource and worth publishing. There are, however, a few points that need to be addressed before I can recommend acceptance in JoVE.*

We thank Reviewer 3 for their comments and feedback. We have addressed each concern below and edits in the revised manuscript are highlighted in grey.

*1. The authors mention that the zebrafish mesonephros has a similar organization than the metanephros (page 3). While generally correct, the authors should point out that there is one big difference and this is the absence of Henle's Loop. Even though there is the argument that the intermediate segment may share some of its functions, this a clear anatomical difference.*

We were remiss in bringing up this point and have revised the introduction accordingly.

*2. There are several issues about the description of the DBA lectin.*

*(2.1) On page 4, in a section on labeling zebrafish nephron segments, the authors mention the use of lectins. But to my knowledge this has not been shown in zebrafish. Thus, citing a manuscript describing DBA in rat is misleading (even if its shown in the subsequent results section) and must be altered.*

We revised these sentences in the introduction to clearly state that DBA labeling is used in mammals, e.g. the rat, to label the collecting ducts.

*(2.2) DBA in mouse and rats labels collecting ducts and is a widely used tool for it. The authors now propose that it labels distal tubules in zebrafish. This in itself is a very valuable piece of information, but it needs to be validated using double labeling. The authors need to show convincingly that it does not label nephric ducts. Just relying on the geometry is not sufficient.*

We have performed double- and triple-labeling combinations to demonstrate that the DBA label is mutually exclusive of the alkaline phosphatase label and dextran uptake (Figures 7 and 8). Further, we demonstrate that distal tubules, which are marked by transcripts encoding *clcnk* (DE and DL segments) are elongated segments that show branched morphologies analogous to DBA-stained tubule regions. We further demonstrate that that the DE, which is stained by transcripts encoding *slc12a1*, is rarely (if at all) branched in shape, while the DL, which is labeled by transcripts encoding the solute transporter *slc12a3,* is commonly found in branched and pinwheel-like morphologies.

*(2.3) Finally the demonstration that there is a difference in the staining pattern of lectins between different organisms is important general knowledge and the authors should discuss this in the discussion section.*

We have added this comment to the discussion.

*3. In fact, double staining is a very important aspect, when studying nephron patterning. It would have been very informative, if some of the methods described include approaches to stain e.g. proximal and distal tubules (plus nuclei).*

Good point. We have added extensive double- and triple-labeling approaches to the revised manuscript. In Figure 6, we show that alkaline phosphatase and dextran uptake partially overlap. Dextran uptake is a widely accepted phenotype of the PCT, and matches the domain of numerous PCT-specific markers (Figures 4, 5). The alkaline phosphatase+/dextran- tubules can only correspond to PST segments because the renal corpuscle flanks the other end of each PCT (see Figure 1). We show that gene transcripts known to be exclusive markers of the PCT and PST are found in directly adjacent domains in the proximal tubule, and that the pattern of the PCT-PST stretch matches the alkaline phosphatase domain (Figure 5). Finally, we show that alkaline phosphatase and DBA are non-overlapping domains, and that the DBA domains correspond to distal tubules that express *clcnk*, and which can be subdivided into the DE and DL based on *slc12a1* and *slc12a3* transcripts, respectively (Figures 6-8).

*4. The discussion is very lengthy and needs to be revamped. The topic of the review is labeling different nephron segments, yet the discussion is mostly about acute kidney injury. While there is little doubt that zebrafish is a valuable model to study kidney disease, this is not part of the manuscripts. A more relevant discussion on nephron organization is needed.*

The discussion has been revamped. We reorganized this section such that the initial two paragraphs provide a relevant discussion on nephron organization, as both you and Reviewer #4 have suggested. Nevertheless, we kept (but revised) the narrative that discusses the scientific context in which to consider the use of adult zebrafish for kidney research. Explaining the state of knowledge about kidney regeneration is vital, as that provides the backdrop for both why and how zebrafish can be used to address deficiencies in our knowledge. At the current time, the major application of the nephron labels presented here is for the characterization of renal regeneration phenotypes. The discussion abides by the standard 3-6 paragraph length for *JoVE* papers.

***Reviewer #4:***

*Manuscript Summary:*

*This paper starts with a cogent and comprehensive review of nephron function, repair, regeneration, and the use of zebrafish as model organism for studying renal injury and repair. The introduction and discussion are interesting to read and well-written but are not particularly focused on the utility of the methods presented in the paper. For example, the discussion contains a paragraph on mechanisms of renal repair in zebrafish, without discussing how or if the methods presented are linked to this topic.*

*The presentation of the methods is clear and the protocols have sufficient details.*

We thank Reviewer 4 for their comments and feedback. We have addressed each concern below and edits in the revised manuscript are highlighted in grey.

*Major Concerns:*

*1. Readers are left to trust the authors about the specificity of the methods. There is no comparison of the claimed region of uptake to independent histological or molecular markers of the relevant tissue segments. The authors do reference marker genes for these regions but do not correlate them with the fluorescence results presented. Such a comparison should be made, or a study showing this comparison should be clearly referenced.*

We have revised the manuscript to provide extensive images to compare the nephron segment labels and correlate them with independent molecular markers, e.g. with gene transcript domains for specific solute transporters that uniquely identify nephron segment populations. For example, dextran uptake is compared to the domain of the PCT in Figure 4. In Figure 5, we provide a series of labels to compare alkaline phosphatase reactivity with the combined domains of the PCT and PST, to make the correlation that alkaline phosphatase is a pan-proximal marker. Next, in Figure 6, we show double labeling of dextran uptake and alkaline phosphatase, demonstrating that they overlap in the PCT and that only alkaline phosphatase labels the PST. Here we show co-labeling by both whole-mount kidney analysis and cryosection analysis—to clearly demonstrate areas of co-expression. In Figure 7, we provide a comparison of alkaline phosphatase with DBA staining, demonstrating that these are mutually exclusive nephron segment regions. Again, we provide whole mount and cryosection data to demonstrate the exclusivity of the alkaline phosphatase and DBA stains. Finally, in Figure 8 we provide triple-labeling data for dextran uptake, alkaline phosphatase, and DBA. These data demonstrate that tubule segments in the adult kidney are double positive for alkaline phosphatase/dextran, positive for alkaline phosphatase alone, or positive for DBA. Based on correlations to molecular features and morphological characteristics of the nephron segments, these combinations demarcate the PCT (alkaline phosphatase/dextran), PST (alkaline phosphatase), and distal tubule (DBA), respectively.

*2. The authors should condense the discussion and focus on the significance, limitation, and utility of the methods presented.*

The discussion abides by the length for *JoVE* papers. We have reorganized and edited the discussion to begin this section with paragraphs focused on the significant, limitations, and utility of the methods presented. We kept the paragraphs that discuss the scientific context in which to consider the use of adult zebrafish for kidney research. Explaining the state of knowledge about kidney regeneration is vital, as that provides the backdrop for both why and how zebrafish can be used to address deficiencies in our knowledge. At the current time, the major application of the nephron labels presented here is for the characterization of renal regeneration phenotypes.