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Quantifying liver size in larval zebrafish using brightfield microscopy

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TITLE:

Quantifying Liver Size in Larval Zebrafish Using Brightfield Microscopy

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KEYWORDS:

Zebrafish, larvae, dissection, liver size, liver analysis, imaging

SUMMARY:

Here we demonstrate a method for quantifying liver size in larval zebrafish, providing a way to assess the effects of genetic and pharmacologic manipulations on liver growth and development.

ABSTRACT:

In several transgenic zebrafish models of hepatocellular carcinoma (HCC), hepatomegaly can be observed during early larval stages. Quantifying larval liver size in zebrafish HCC models provides a means to rapidly assess the effects of drugs and other manipulations on an oncogene-related phenotype. Here we show how to fix zebrafish larvae, dissect the tissues surrounding the liver, photograph livers using bright-field microscopy, measure liver area, and analyze results. This protocol enables rapid, precise quantification of liver size. As this method involves measuring liver area, it may underestimate differences in liver volume, and complementary methodologies are required to differentiate between changes in cell size and changes in cell number. The dissection technique described herein is an excellent tool to visualize the liver, gut, and pancreas in their natural positions for myriad downstream applications including immunofluorescence staining and in situ hybridization. The described strategy for quantifying larval liver size is applicable to many aspects of liver development and regeneration.

INTRODUCTION:

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver[1] and the third leading cause of cancer-related mortality[2]. To better understand mechanisms of hepatocarcinogenesis and identify potential HCC therapeutics, we and others have developed transgenic zebrafish in which hepatocyte-specific expression of oncogenes such as β-catenin[3,4],

Kras(V12)[5,6], Myc[7], or Yap1[8] leads to HCC in adult animals. In these zebrafish, liver enlargement is noted as early as 6 days post fertilization (dpf), providing a facile platform for testing the effects of drugs and genetic alterations on oncogene-driven liver overgrowth. Accurate and precise measurement of larval liver size is essential for determining the effects of these manipulations.

Liver size and shape can be assessed semi-quantitatively in fixed zebrafish larvae by CY3-SA labeling[9] or in live zebrafish larvae using hepatocyte-specific fluorescent reporters and fluorescence dissecting microscopy[5,6]. The latter method is relatively quick, and its lack of precision can be addressed by photographing and measuring the area of each liver using image processing software[7,10]. However, it can be technically challenging to uniformly position all live larvae in an experiment such that two-dimensional liver area is an accurate representation of liver size. A similar technique for quantifying liver size involves using light sheet fluorescence microscopy to quantify larval liver volume[8], which may be more accurate for detecting size differences when the liver is expanded non-uniformly in different dimensions. Fluorescence-activated cell sorting (FACS) can be used to count the number of fluorescently labeled hepatocytes and other liver cell types in larval livers[8,11]. In this method, larval livers are pooled and dissociated, so information about individual liver size and shape is lost. In combination with another liver size determination method, FACS enables differentiation between increased cell number (hyperplasia) and increased cell size (hypertrophy). All of these methods employ expensive fluorescence technology (microscope or cell sorter) and, except for CY3-SA labeling, require labeling of hepatocytes with a fluorescent reporter.

Here we describe in detail a method for quantifying zebrafish larval liver area using bright-field microscopy and image processing software[3,12–14]. This protocol enables precise quantification of the area of individual livers in situ without the use of fluorescence microscopy. While analyzing liver size, we blind the image identity to reduce investigator bias and improve scientific rigor[15].

PROTOCOL:

Animal studies are carried out following procedures approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Utah.

1. Fixing larvae

1.1. At 3–7 days post fertilization (dpf), euthanize larvae with tricaine methanesulfonate (0.03%) and collect up to 15 larvae in a 2 mL tube using a glass pipette and pipette pump.

1.2. Wash larvae twice with 1 mL of cold (4 °C) 1x phosphate-buffered saline (PBS) on ice. For each wash, remove as much liquid as possible from the tube with a glass pipette and pipette pump, and then add 1 mL of cold PBS to tube.

1.3. Remove as much PBS as possible using a glass pipette and pipette pump, and add 1 mL of cold (4 °C) 4% paraformaldehyde (PFA) in PBS.

CAUTION: PFA is an irritant and suspected carcinogen. Gloves should be worn when handling PFA, and concentrated solutions should be handled in a chemical fume hood.

1.4. Incubate at 4 °C at least overnight (but up to several months) with gentle rocking.

2. Dissecting tissues surrounding liver

2.1. Remove larvae from PFA by rinsing 3x with 1 mL of cold (4°C) PBS and rocking for 5 min in between rinses.

NOTE: It is okay to keep the rinsed larvae in PBS for a day or two at 4 °C.

2.2. Pipette several larvae in PBS into one well of a 9-well round-bottom glass dish.

2.3. Remove skin surrounding liver.

2.3.1. Use fine forceps to hold larva on its back (belly up), gripping on either side of the head as gently as possible. Then use very fine forceps in your other hand to grab the skin just overlying the heart.

2.3.2. Pull skin down diagonally towards the tail of the fish and the bottom of the dish on the left or right side of the fish. Repeat for other side (right or left side).

2.3.3. Continue grabbing flaps of skin and pulling down/back until all of the skin and melanophores overlying or near the liver have been removed.

2.4. Remove yolk, if present.

2.4.1. For 5–6 dpf larvae, lift yolk off in one piece by holding the fish with fine forceps on its back and using the very fine forceps to prod the yolk gently.

2.4.2. For 3–4 dpf larvae, scrape the yolk off in pieces. Hold the fish with fine forceps on its back and use the very fine forceps to stroke the yolk, starting from the ventral side.

2.5. Place dissected larvae into fresh cold PBS using a glass pipette and pipette pump.

3. Imaging

3.1. To mount larvae, pour a few mL of 3% methyl cellulose onto the lid of a clean plastic Petri dish.

3.2. Use a glass pipette and pipette pump to add larvae to the methyl cellulose, adding as little PBS with the larvae as possible.

3.3. Under a dissecting microscope at low magnification, use fine forceps to orient the fish so they are laying on their right side, facing left.

NOTE: Make sure the fish are oriented perfectly on their side or the liver measurements may not be accurate.

3.4. Take a picture of each fish.

3.4.1. Confirm that the fish to be photographed is aligned perfectly, with one eye directly on top of the other eye. If necessary, use fine forceps to tap lightly on head or tail of fish to adjust orientation. If fish's tail is bent, remove the tail by pinching it forcefully with forceps to remove it so the fish lays flat.

3.4.2. Zoom in to high magnification and focus on the liver, making sure that the liver's outline is clearly visible.

3.4.3. Snap a picture and save the file.

3.4.4. Repeat for all fish, making sure the magnification is the same for each picture.

3.4.5. Take a picture of a micrometer using the same magnification (see **Figure 2H**).

4. Image analysis

4.1. Measure the area of each fish's liver using image processing software.

4.1.1. Blind all liver pictures to avoid potential investigator bias and promote scientific rigor[15]. This step can be done manually by another lab member or using a computer program (Supplementary Material). Rename files randomly and create a "randomization file" containing a list of the original file names and corresponding blinded file names.

4.1.2. Open randomized files in order, starting with file 1.

4.1.3. Choose the freehand selections tool and outline each liver.

4.1.4. Press Ctrl-M to measure the area of each liver.

4.1.5. For any livers that cannot be accurately measured, insert a placeholder measurement (very small or very large, so it can be easily excluded later on).

4.1.6. Save the measurements in a text file ("measurements file").

4.2. Un-blind and analyze data

4.2.1. Open “measurements file” and “randomization file” in a spreadsheet program.

4.2.2. Insert a new column in the “measurements file” and add the original file names for the blinded files, using the “randomization file”. Save this file as “unblinded measurements file”.

4.2.3. Sort data by original file name.

4.2.4. Be sure to exclude any liver measurements for which pictures were inadequate (see **Figure 2A–G**).

4.2.5. If necessary, convert measurement values into desired scale (mm^2, for example).

4.2.5.1. Open the scale bar in the image processing software.

4.2.5.2. Use the straight line tool to measure 1 mm on the scale bar. The image processing software will measure in the same units as the livers (pixels), giving a conversion factor.

4.2.5.3. Use the conversion factor to convert measurements in the “unblinded measurements file”.

4.2.6. Using the spreadsheet program or pasting data into a scientific graphing and statistics software, determine mean and standard deviation and calculate *p* value(s).

REPRESENTATIVE RESULTS:

Transgenic zebrafish expressing hepatocyte-specific activated β-catenin (*Tg(fabp10a:pt-β-cat)* zebrafish)[3] and non-transgenic control siblings were euthanized at 6 dpf and liver area was quantified using brightfield microscopy and image processing software. Transgenic zebrafish have significantly increased liver size (0.0006 cm^2) as compared to their non-transgenic siblings (0.0004 cm^2, $p < 0.0001$; **Figure 1**).

FIGURE AND TABLE LEGENDS:

Figure 1: Liver size analysis of 6 dpf (days post fertilization) zebrafish. (A-B) Representative brightfield image of 6 dpf non-transgenic zebrafish larva, which shows natural position and shape of liver overlying the gut. Liver area has been outlined in **(B)**. Scale bars indicate 0.1 mm. **(C-D)** Representative brightfield image of 6 dpf transgenic zebrafish larva expressing hepatocyte-specific activated β-catenin (ABC), showing enlarged liver. Liver area has been outlined in **(D)**. Scale bars indicate 0.1 mm. **(E)** Graph showing liver size measurements (mean ± standard deviation) of 6 dpf non-transgenic zebrafish larvae (Non-Tg) and transgenic zebrafish larvae expressing hepatocyte-specific activated β-catenin (ABC). Samples were compared using unpaired t test. ****$p < 0.0001$.

Figure 2: Examples of inadequate images and micrometer. (A-G) Representative images of larval livers that should be excluded from analysis. Scale bars indicate 0.1 mm. **(A)** Larva with skin

covering the liver. **(B)** Larva with yolk obscuring the liver. **(C)** Larva with parts of the liver pinched off. **(D)** Larva with liver dislocated and falling off. **(E)** Larva with missing liver. **(F)** Larva with liver outline that is difficult to identify because image is blurred/out-of-focus. **(G)** Larva with improper positioning. The two eyes are not aligned directly on top of each other. **(H)** Image of the micrometer, used to generate scale bars and convert image processing software measurements from pixels to cm^2**.**

DISCUSSION:

Quantification of liver size is crucial in studies aimed at understanding liver development, regeneration, and oncogenesis. The protocol described here is a relatively quick, easy, and cheap technique for liver size quantification in larval zebrafish. Exercising appropriate caution while performing certain aspects of the protocol can aid in increased accuracy of results and decreased frustration.

Proper fixation of the larvae is crucial towards getting well-preserved biological samples and preventing their disintegration. Dilution of the 4% PFA solution can occur when PBS is not removed completely before the addition of PFA to the rinsed larvae. Using well-made PFA solutions and pipetting out all or most of the PBS solution prior to PFA addition is helpful to address this issue.

Although fast and easy to perform after much practice, the dissection technique requires substantial manual dexterity. While dissecting, it is crucial to remove the skin and yolk completely off from above the liver such that the whole liver is exposed. Failure to do this can result in images where the view of the liver boundary is obscured (**Figure 2A,B**). Unskilled and forceful movements while dissecting can lead to pinching off of parts of the liver (**Figure 2C**) or loosening of liver attachments, resulting in the liver being displaced (**Figure 2D**) or missing entirely (**Figure 2E**). Users should put in adequate numbers of hours towards honing their dissecting skills on practice samples before moving on to experimental samples.

During mounting, the skin above the liver has been removed, increasing the probability of the liver falling out during subsequent steps. To avoid that possibility, gentle pipetting movements should be employed during this process.

During image procurement using the brightfield microscope, it is crucial that good quality images are taken. Blurry, out-of-focus images will make it difficult to assess the true boundary of the liver (**Figure 2F**). As this method involves measuring the surface area of the left lobe of the liver, it is crucial that the larva is oriented well on its side and not tilted (**Figure 2G**). Make sure that both eyes of the larva are aligned (one eye covering the view of the other). While measuring surface area using image processing software, it is important to draw the boundary as close as possible to the real outline of the liver so as to avoid measurement discrepancies. Exclude any images where the liver cannot be accurately measured (**Figure 2A–G**). However, keep in mind that excluding livers can skew the data, as bigger livers are more likely to be disrupted than smaller livers.

One of the limitations of this protocol is that it applies only to fixed larvae. Alternative methods such as fluorescence microscopy can be used to measure liver size in live larvae expressing hepatocyte-specific fluorescent reporters[5,7,10]. These alternative methods enable sequential measurements to be made on the same animal, and they are also quicker, since they do not require fixation or dissection of the tissues overlying the liver. The advantages of this protocol compared to fluorescence microscopy in live animals are: 1) more flexibility with respect to when livers are measured, as zebrafish can be kept in fixative for weeks or months before photographing them; 2) no requirement for incorporating a fluorescent reporter, which can be cumbersome when dealing with homozygous mutants; and 3) applicability of steps 1 and 2 for other experiments, including immunofluorescence staining or in situ hybridization studies. We use both methods, depending on the particular application. For example, we typically use live imaging and hepatocyte-specific reporters for high-throughput screening[3], and follow up on potential hit compounds using the protocol described here[3].

This protocol takes only the surface area into account for quantification of liver size, so it does not detect changes in cell metabolism or morphology, nor does it differentiate between increases in cell number and increases in cell size. In order to address this limitation, complementary assays to assess steatosis[16], histology[6], cell number[8,11], cell size[3,17], proliferation[3,18], and/or apoptosis[19] can be performed.

Another limitation of this protocol is that it assumes that increases or decreases in the surface area of the left liver lobe are reflective of the changes in surface area and volume of liver as a whole. This assumption may not apply when liver growth is non-uniform. To examine liver shape and check for non-symmetric increases in liver growth, we routinely do light sheet fluorescence microscopy[8] or confocal microscopy[3] on our transgenic models. Light sheet fluorescence microscopy can be used to directly quantify larval liver volume[8]. In transgenic zebrafish expressing hepatocyte-specific Yap1, liver area and liver volume were similarly increased compared to non-transgenic control siblings[8].

The dissection technique described here can be combined with immunofluorescence staining, cell-specific fluorescent reporter lines, and/or other labeling techniques to study other aspects of liver development besides liver size[3,19,20]. As this dissection protocol also exposes the gut and pancreas, it may be helpful for studies of other visceral organs as well.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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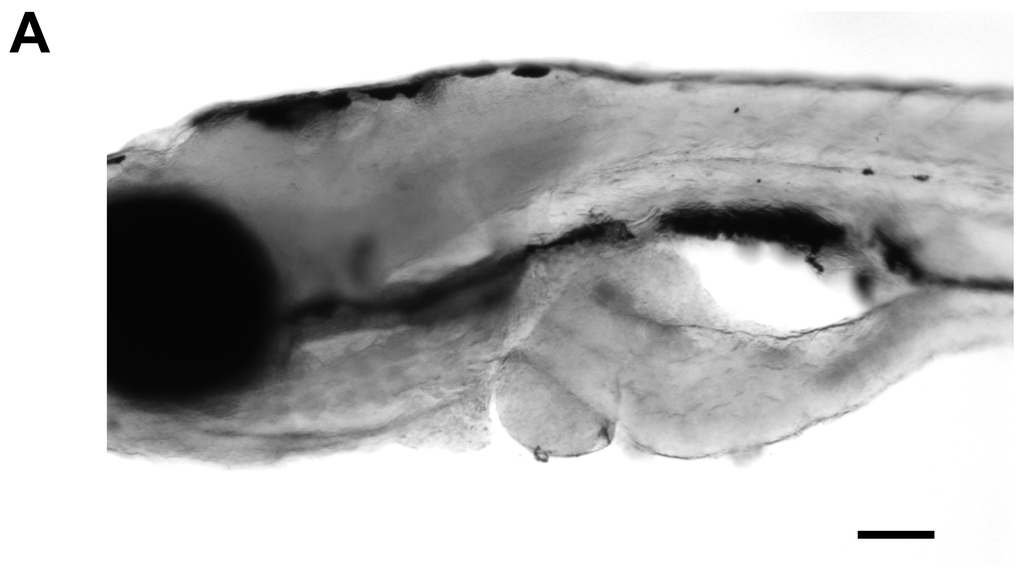

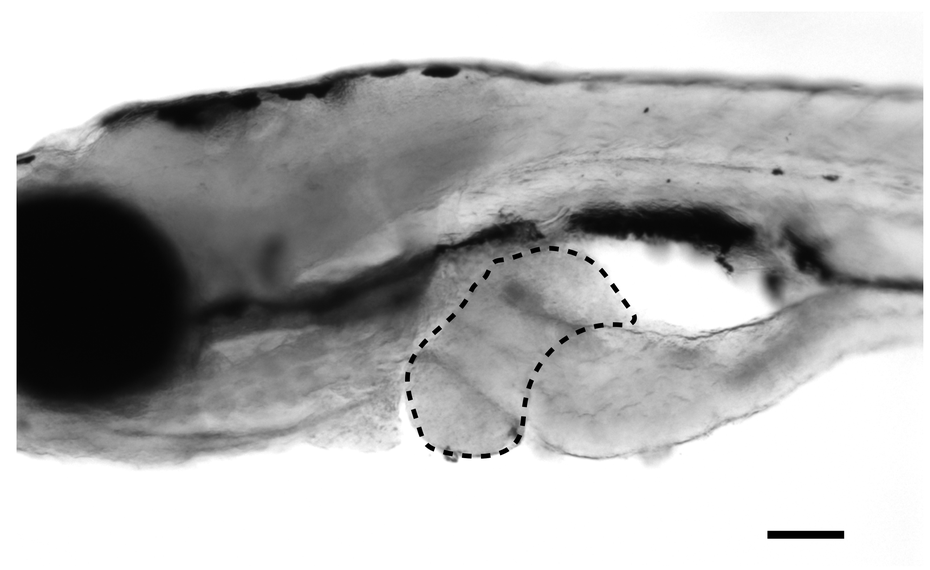

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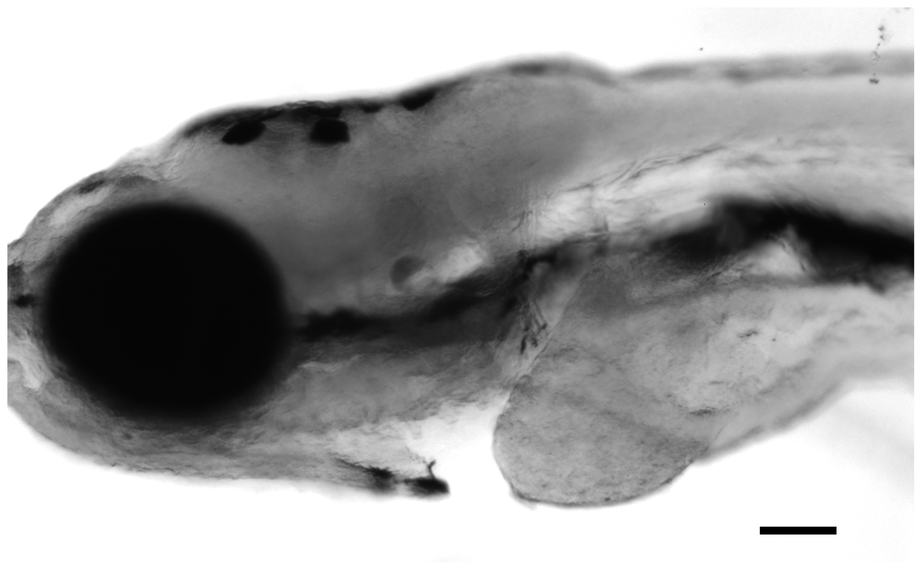
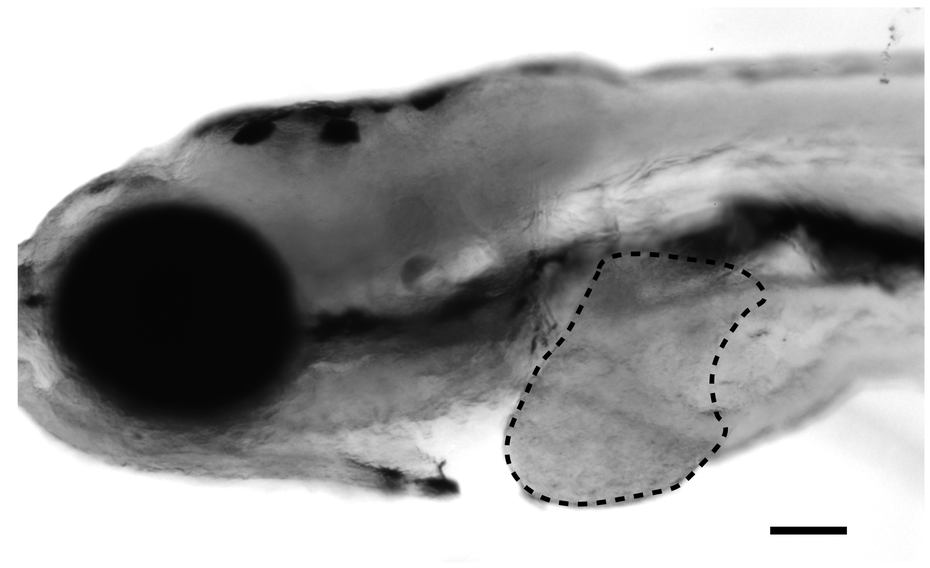
Non-transgenic





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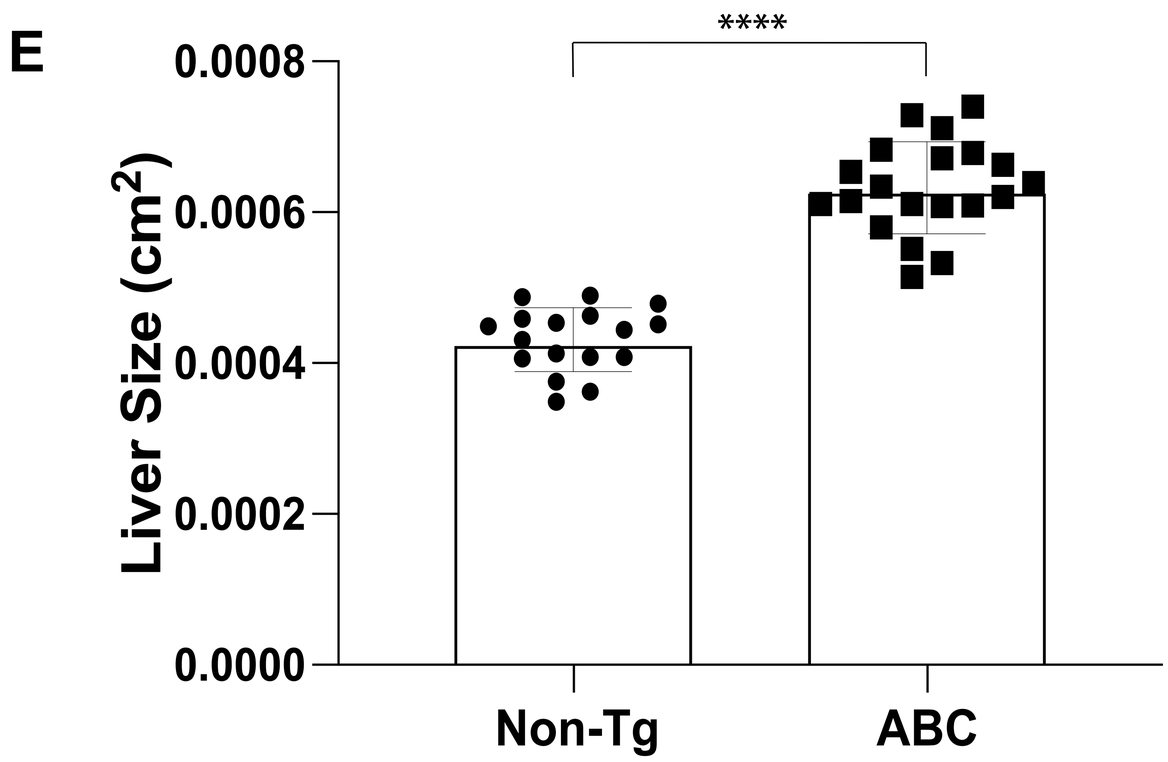

Figure 2

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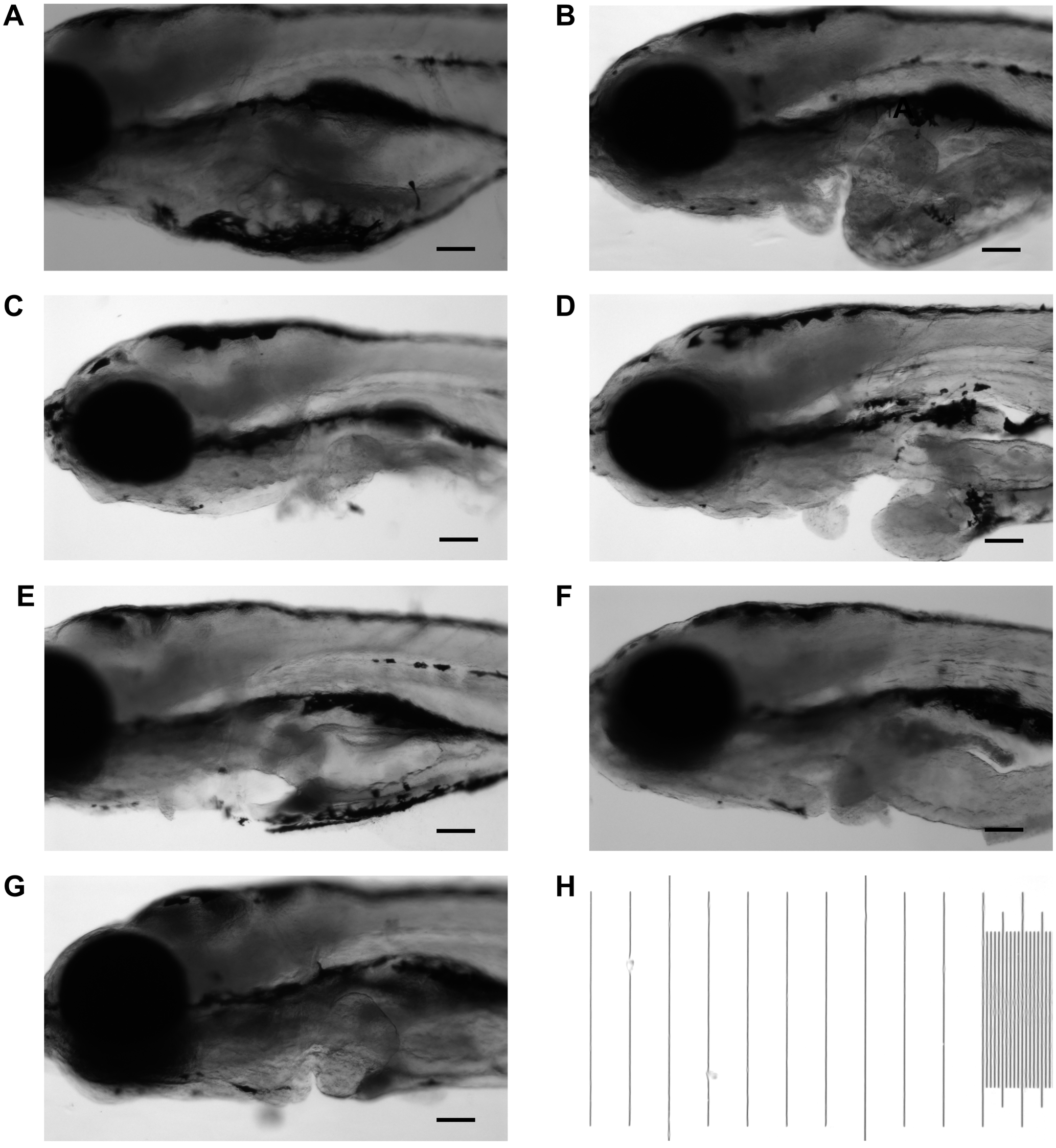

Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Camera for dissecting microscope	Leica, for example		
Dissecting microscope	Leica, for example		
Fine (Dumont #5) forceps	Fine Science Tools	11254-20	
Glass pipets	VWR	14672-608	
Image analysis software	Image J/FIJI		ImageJ/FIJI can be dowloaded for free
Methyl cellulose	Sigma	M0387	
Paraformaldehyde	Sigma Aldrich	P6148	
Phosphate-buffered saline	Various suppliers		
Pipette pump	VWR	53502-233	
Plastic Petri dishes	USA Scientific Inc	2906	
Pyrex 9-well round-bottom glass dish	VWR	89090-482	
Software for blinding files	R project		R can be downloaded for free: https:/
Scientific graphing and statistics software	GraphPad Prism		
Spreadsheet program	Microsoft Excel		
Tricaine methanesulfonate (Tricaine-S)	Western Chemical	200-226	
Very fine (Dumont #55) forceps	Fine Science Tools	11255-20	

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We thank the editor and reviewers for their helpful comments. We have addressed their specific concerns as detailed below:

Editorial comments:
General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
This has been done.

2. Please include at least 6 key words or phrases.
This has been done (zebrafish, larvae, dissection, liver size, liver analysis, imaging)

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Pyrex, Excel, GraphPad Prism
Commercial language has been removed from the text.

Protocol:
1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.
This has been done. We added substeps for step 4.1.1.

Specific Protocol steps:
1. 4.1.1: Please provide more information and/or include example code as supplementary material.
More information has been added (substeps 4.1.1.1 and 4.1.1.2) to explain how to blind/randomize files either manually or using a computer program. Example R code has been included as Supplementary Material.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.
This has been done.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript describes the method for fixing zebrafish larvae, dissection technique removing the tissues surrounding the liver, photograph livers using bright-field microscopy, measure liver area with Image J, and analyze results. This protocol enables precise quantification of liver size in zebrafish larva using brightfield microscopy.
Fixing: 3-7 days post fertilization (dpf), euthanize larvae with tricaine methanesulfonate (0.03%), wash with PBS and fix with 1 mL of cold (4C) 4% paraformaldehyde (PFA) in PBS.
Dissecting: Remove skin surrounding liver, this surgical technique required high dexterity and well-trained person, "Use fine (Dumont #5) forceps to hold larva on its back

(belly up), gripping on either side of the head as gently as possible. Then use very fine (Dumont #55) forceps in your other hand to grab the skin just overlying the heart." Imaging: this surgical technique required high dexterity and well-trained person, "Use a glass pipette to add larvae to the methyl cellulose, under a dissecting microscope at low magnification, use fine forceps to orient the fish so they are laying on their right side, facing left."
Image analysis: There are two steps: 1. Measure the area of each fish's liver using ImageJ/FIJI-Randomize/blind all liver pictures using R. 2. Un-blind and analyze data, Be sure to exclude any liver measurements for which pictures were inadequate, Using Excel or pasting data into GraphPad Prism, determine mean and standard deviation and calculate P value(s).

Major Concerns:

1. Many methods have been established to observe the alteration of liver in zebrafish larvae. Dai et al. defined the steatosis in zebrafish larvae by using oil red O staining and bright-field microscope without any surgical procedure (1). As authors also already mentioned in the introduction and discussion, liver specific fluorescent reporters also have been used to study the liver size change. Nguyen et al. used this method before to determine the size of liver (2). What is the advantage of the current protocol compared with the previous method? What is the necessity of tissues dissection procedure? Is there any comparison of the precision using dissection procedure and direct imaging? According to lines "240-251" this surgical technique required high dexterity and well-trained person, otherwise the results will be defected. This method requires more times with surgical procedure for each larva, is it still applicable for high-throughput screening aspect?

We agree that many methods have been established to quantify alterations of zebrafish larval livers. The oil red O staining method examines lipid accumulation in the liver, so this protocol would be a useful complementary approach for looking at metabolic changes. We have added the Dai *et al.* reference to the discussion section. Using liver-specific fluorescent reporters in live zebrafish to quantify liver size has advantages and disadvantages over the current protocol. It enables sequential measurements to be made on the same animal, and it is also quicker, since it does not require fixation or dissection of the tissues overlying the liver. The advantages of this protocol compared to fluorescence microscopy in live animals are: 1) more flexibility with respect to when livers are measured, as zebrafish can be kept in fixative for weeks or months before photographing them; 2) no requirement for incorporating a fluorescent reporter, which can be cumbersome when dealing with homozygous mutants; and 3) applicability of steps 1 and 2 for other experiments, including immunofluorescence staining of the liver, gut, and pancreas. We have added the Nguyen reference to the introduction and this additional discussion to the discussion section.

The dissection procedure is required in order to see the boundary of the liver well and take precise measurements. Without dissection (or with incomplete dissection), the boundary of the liver is obscured (Figure 2A,B).

We have done live imaging ourselves and found it to be less precise than the current protocol. However, we don’t have a direct comparison of the two methods using the same animals.

We do not think this is the most suitable method for high-throughput screening. We added the following sentences to the discussion: We use both methods, depending on the particular

application. For example, we typically use live imaging and hepatocyte-specific reporters for high-throughput screening, and follow up on potential hit compounds using the protocol described here.

2. What is the importance of blind and un-blind files in the lines "158-178"? Did authors have conflict of interest / subjective opinion that could affect the measurement?

To promote optimum rigor and reproducibility in animal studies, it is important that a core set of standards is followed (Landis *et al,* 2012). This core set of standards includes randomization, blinding, sample-size estimation, and data handling (Landis *et al,* 2012). Blinding is important to reduce conscious or unconscious investigator bias regarding the potential outcome of each experiment. We have added a reference describing the importance of blinding to scientific rigor (Landis *et al*. 2012).

3. Please describe more detail about statistical analysis that authors used for Figure 1E. What kind of statistical analysis did authors use? Please put asterisk on the graph.

In Figure 1E, samples were compared using unpaired T-test ($p<0.0001$). There is now an asterisk on the graph.

It is important to compare this protocol with other previous protocols and showed the advantages of dissecting removing the tissues surrounding the liver, and Image analysis using Randomize/blind all liver pictures and Un-blind and analyze data.

Thank you for your helpful suggestions. We have addressed each of these important issues as described above.

References

1. Dai W, Wang K, Zheng X, Chen X, Zhang W, Zhang Y, et al. High fat plus high cholesterol diet lead to hepatic steatosis in zebrafish larvae: a novel model for screening anti-hepatic steatosis drugs. Nutrition & metabolism. 2015;12(1):42.
2. Nguyen AT, Emelyanov A, Koh CHV, Spitsbergen JM, Parinov S, Gong Z. An inducible krasV12 transgenic zebrafish model for liver tumorigenesis and chemical drug screening. Disease models & mechanisms. 2012;5(1):63-72.

Reviewer #2:
Manuscript Summary:
This manuscript describes a simple and inexpensive method for measuring zebrafish larval liver area using brightfield microscopy and ImageJ. In several zebrafish models of hepatocellular carcinoma (HCC), increases in larval liver size correlate with adult development of HCC. This method uses area of the left liver lobe as a surrogate for liver volume, which requires more expensive and time-consuming methods for measurement. The distinction between these methods and potential drawbacks of measuring only area are described in the manuscript. Figures demonstrate the method for measuring liver volume, and several technical pitfalls that can preclude this method.

Major Concerns:
None

Minor Concerns:

Discussion, lines 266-270: the authors are careful to note that measuring the area of the left liver lobe may not reflect changes in liver volume if growth is not symmetrical. sI would like a bit more discussion of this point. For example, how would one determine if a larval zebrafish line has non-symmetric increases in liver growth? Do they routinely check this in their transgenic models? Also, if they also use a fluorescent method to measure liver volume (lightsheet microscopy, for example), have they measured area and volume in the same larvae to compare differences? How sensitive is the method described here as compared to volume-based measurements?

Thanks for your suggestion. We have added additional discussion on this point. To examine liver shape and check for non-symmetric increased in liver growth, we routinely do light sheet fluorescence microscopy or confocal microscopy on our transgenic models. We have not directly compared a fluorescent method to this method in the same larvae, but we have measured area and volume in different clutches from the same transgenic line. In transgenic zebrafish expressing hepatocyte-specific Yap1, liver area and liver volume were similarly increased compared to non-transgenic control siblings.

Reviewer #3:
Srishti Kotiyal et al used light microscopy and ImageJ to quantify the liver size in larval zebrafish. Zebrafish is one of the popular vertebrate models to study liver development, regeneration, and cancer. Although there are a number of methods to quantify the liver size including the fluorescence in transgenic lines, immunofluorescent staining, and in situ hybridization, quantification of the liver size in bright filed is limited. Srishti Kotiyal et al used 4% PFA to fix the larvae and then removed the skin and yolk by forceps manually, which avoid the false positive to outline the liver region. This protocol described here is a relatively quick, easy, and cheap technique for liver size quantification in larval zebrafish, and the experimental procedure is clear and concise. I anticipate that this manuscript will be very well received by the JoVE community. I recommend acceptance of the manuscript.
Thank you for your positive comments. We hope that this manuscript will be helpful to the JoVE community.

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