Journal of Visualized Experiments

Using zebrafish larvae to study the pathological consequences of haemorrhagic stroke

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59716R2
Full Title:	Using zebrafish larvae to study the pathological consequences of haemorrhagic stroke
Keywords:	intracerebral haemorrhage; Stroke; zebrafish; brain injury; locomotor; neuroinflammation
Corresponding Author:	Paul Kasher The University of Manchester Manchester, Greater Manchester UNITED KINGDOM
Corresponding Author's Institution:	The University of Manchester
Corresponding Author E-Mail:	paul.kasher@manchester.ac.uk
Order of Authors:	Siobhan Crilly
	Alexandra Njegic
	Adrian Parry-Jones
	Stuart Allan
	Paul Kasher
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Manchester, Greater Manchester, United Kingdom

TITLE:

Using Zebrafish Larvae to Study the Pathological Consequences of Hemorrhagic Stroke

AUTHORS & AFFILIATIONS:

Siobhan Crilly[1], Alexandra Njegic[2], Adrian R. Parry-Jones[2], Stuart M. Allan[1], Paul R. Kasher[1]

[1]Division of Neuroscience and Experimental Psychology, School of Biological Sciences, Manchester Academic Health Science Centre, University of Manchester, Michael Smith Building, Oxford Road, Manchester, UK

[2]Division of Cardiovascular Sciences, School of Medical Sciences, Faculty of Biology, Medicine and Health, Manchester Academic Health Science Centre, University of Manchester, Michael Smith Building, Oxford Road, Manchester, UK

Corresponding author:

Paul R Kasher (paul.kasher@manchester.ac.uk)

Telephone: +44 161 306 8059

Email Addresses of Co-Authors:

Siobhan Crilly (siobhan.crilly@manchester.ac.uk)

Alexandra Njegic (alexandra.njegic@postgrad.manchester.ac.uk)

Adrian R. Parry-Jones (adrian.parry-jones@manchester.ac.uk)

Stuart M. Allan (stuart.allan@manchester.ac.uk)

KEYWORDS:

Intracerebral hemorrhage, zebrafish, brain injury, stroke, neuroinflammation, animal models, pre-clinical.

SUMMARY:

Here we present a protocol to quantify brain injury, locomotor deficits and neuroinflammation following bleeding in the brain in zebrafish larvae, in the context of human intracerebral hemorrhage (ICH).

BSTRACT:

Despite being the most severe subtype of stroke with high global mortality, there is no specific treatment for patients with intracerebral hemorrhage (ICH). Modelling ICH pre-clinically has proven difficult, and current rodent models poorly recapitulate the spontaneous nature of human ICH. Therefore, there is an urgent requirement for alternative pre-clinical methodologies for study of disease mechanisms in ICH and for potential drug discovery.

The use of zebrafish represents an increasingly popular approach for translational research, primarily due to a number of advantages they possess over mammalian models of disease, including prolific reproduction rates and larval transparency allowing for live imaging. Other groups have established that zebrafish larvae can exhibit spontaneous ICH following genetic or chemical disruption of cerebrovascular development. The aim of this methodology is to utilize

such models to study the pathological consequences of brain hemorrhage, in the context of pre-clinical ICH research. By using live imaging and motility assays, brain damage, neuroinflammation and locomotor function following ICH can be assessed and quantified.

This study shows that key pathological consequences of brain hemorrhage in humans are conserved in zebrafish larvae highlighting the model organism as a valuable in vivo system for pre-clinical investigation of ICH. The aim of this methodology is to enable the pre-clinical stroke community to employ the zebrafish larval model as an alternative complementary model system to rodents.

INTRODUCTION:

Intracerebral hemorrhage (ICH) is the most severe sub-type of stroke associated with spontaneous cerebral vessel rupture and bleeding into the parenchyma leading to brain damage, physical disability and often death[1]. Despite the high mortality and morbidity rate associated with ICH[2], understanding of the underpinning etiology and post-hemorrhage pathology is still lacking. As such, there are no specific treatments to prevent ICH or improve patient outcomes. Most of our understanding of disease biology has come from pre-clinical rodent models of ICH[3], however studies to-date in these models have failed to translate any successful therapeutic to the clinic[4,5]. This failure may be due in part, to some limitations of these preclinical models, including the inability to easily recapitulate the spontaneous nature of human disease and the requirement for invasive surgery to generate the models in mammals[6]. Additionally, rodents pose practical problems with regards to observing the rapid onset of cellular responses to ICH in intact tissue. Given the lack of translation from rodent models, developing alternative models of spontaneous ICH is imperative if we are to overcome these practical problems and help identify novel drug targets.

The molecular mechanisms of vascular development are well conserved amongst vertebrates including zebrafish (*Danio rerio*)[7]. As such, the adoption of this model organism is becoming an ever more useful mechanistic strategy for studying cerebrovascular disease[8]. A number of zebrafish models have been generated which recapitulate phenotypes associated with stroke-related conditions[9-12]. The use of zebrafish larvae to investigate disease pathogenesis offers both practical and scientific advantages over mammalian models[8]. This includes high reproduction rates, rapid development and larval transparency that allows for intravital imaging without the invasive constraints associated with rodents. Coupling these advantages with the wide range of transgenic reporter lines available within the zebrafish research community amounts to a powerful *in vivo* approach for studying disease biology, not yet utilized for studying the pathological consequences of ICH.

The injury response to blood in the brain is biphasic[13]; the primary insult causes neuronal death and cell necrosis, which then initiates a secondary wave of damage that is induced by innate immune activation. The second phase of brain injury, in particular the neuroinflammatory component, is considered a realistic target for future drug treatment[13]. Spontaneous and cerebral-specific hemorrhages have been described in zebrafish larvae previously[14-19]. Two such models are the use of atorvastatin (ATV) at 24 h post-fertilization (hpf) to inhibit the HMGCR pathway and cholesterol biosynthesis[14], and a bubblehead (bbh) mutant which express a

hypomorphic mutation in the *arhgef7* gene, βpix, and subsequently inhibits actin remodeling for tight endovascular junctions[18]. These models exhibit spontaneous cerebral-specific blood vessel rupture at the onset of circulation (~33 hpf). Recently, we have characterized these models further to reveal that key aspects of the brain injury response is conserved between humans and zebrafish larvae[20]. This study demonstrates the methodology required to obtain and visualize spontaneous brain hemorrhages in zebrafish larvae and how to quantify brain injury, and locomotor and neuroinflammatory phenotypes that relate to the human condition. These data and techniques support the use of this model species as a valuable complementary system for pre-clinical ICH research.

PROTOCOL:

Zebrafish were raised and maintained at The University of Manchester Biological Services Unit under standard conditions as previously described[21]. Adult zebrafish husbandry was approved by the University of Manchester Animal Welfare and Ethical Review Board. All experiments were performed in accordance with U.K. Home Office regulations (PPL:P132EB6D7).

NOTE: Transgenic lines used in this study include macrophage-specific lineage *mpeg1:*mCherry (constructed in-house as previously described[22]), neutrophil-specific *mpo:*GFP[23], erythroid-specific *gata1:*dsRed[24] and *ubiq:*secAnnexinV-mVenus, a reporter for cell death (re-derived in house[25]) on wild-type, nacre (*mitfa*$^{w2/w2}$) and mutant (*bbh*^{m292}) backgrounds. **Figure 1** shows the experimental timeline.

[Place figure 1 here]

1. Day 0: Egg production and collection

1.1. Collect fertilized embryos from natural spawning in breeding boxes produced from 1 male and 1–2 female adult zebrafish.

NOTE: For atorvastatin protocol any wildtype/transgenic animals can be used however hemorrhage rates differ slightly between strains.

1.2. Incubate 100 embryos at 28 °C in standard E3 embryo medium per Petri dish and stage according to standard guidelines[26].

1.3. At ~6 hours post fertilization (hpf) remove dead and unfertilized embryos from the dish using a Pasteur pipette.

2. Day 1: Atorvastatin treatment at 24 hpf

2.1. Dechorionate embryos for atorvastatin treatment using sharp ultra thin dissection forceps[27]. Numbers required for experimentation can be adjusted accordingly.

2.2. Add 30 mL of E3 embryo medium to two clean Petri dishes. Use one dish for 100 embryos.

NOTE: If plates are designed for cell culture, dechorionated zebrafish at this early stage often stick to the bottom. To avoid this, rinse the plates thoroughly in clean water before use.

2.3. Remove 60 µL of embryo water from the treatment plate and add 60 µL of 0.5 mM atorvastatin (ATV). At a 0.5 mM stock concentration, the above dilution will result in a final concentration of 1 µM which will result in ~20% of larvae non-hemorrhaged (ICH-) and ~80% of larvae hemorrhaged (ICH+). Use the other plate for untreated controls

NOTE: Atorvastatin is solubilized in distilled water (3 mg into 10 mL) to make a 0.5 mM stock solution. Incubate overnight at room temperature in the dark with agitation as solubilization takes some time. Complete solubilization can take up to 1 week. Do not use DMSO. Solution is aliquoted and stored at -20°C. Do not freeze thaw.

2.4. Using a Pasteur pipette, transfer 100 embryos in as little water as possible to the treatment plates.

2.5. Incubate the plates at 28 °C.

NOTE: ICH will occur between 33 and 48 hpf. Atorvastatin does not need to be removed as incubation longer than 24 h does not cause any further developmental issues.

3. Day 2: Separating ICH- and ICH+ populations at 50 hpf

3.1. Separate ICH+ fish from ICH- populations and transfer to new dishes for ease.

3.1.1. If using the ATV model at a concentration of 1 µM, 75–100% of larvae will exhibit hemorrhage (ICH+) at this time point.

NOTE: The response of the larvae differs between strains, if larvae are not hemorrhaging at the desired frequencies, use a fresh batch of atorvastatin or a higher concentration. If larvae have not exhibited hemorrhage by 48 hpf then consider them ICH-.

3.1.2. If using the bbh model, all homozygous mutants will exhibit hemorrhage by 48 hpf. If using a heterozygous incross, the ICH- heterozygous and wildtype siblings can be used as control animals for experiments.

3.2. If necessary, anaesthetize the larvae by adding 0.02% MS222 to the E3 media. Using a Pasteur pipette, sort the larvae for presence of blood in the head into fresh E3 media. See **Figure 2**.

NOTE: Blood in the head may appear in the fore, mid or hindbrain or in combination, and bleed volume can vary between animals. In the bbh mutants, ICH is often associated with severe edema recognizable by larger heads, wider space between the eyes and a more diffuse bleed. However not all ICH+ bbh larvae exhibit edema.

[Place figure 2 here]

4. Day 3: Cell death and leukocyte analysis at 72 hpf

4.1. Screen the larvae using a fluorescence microscope to ensure the expression of fluorescent protein.

NOTE: In this study, transgenic *ubiq*:secAnnexinV-mVenus larvae were used to report brain cell death and double transgenic *mpo*:GFP;*mpeg1*:mCherry or *ubiq*:secAnnexinV-mVenus;*mpeg1*:mCherry used for leukocyte analysis.

4.2. Fill the lightsheet mounting chamber with E3 media containing 0.02% MS222.

4.3 Anaesthetize the larvae using 0.02% MS222. Transfer larvae for mounting (n = 1–6) to a dry Petri dish surface in a single droplet. Remove as much liquid as possible.

NOTE: 1.5% low melt agarose is prepared using 0.15 g of low melt agarose dissolved in 10 mL of E3 medium without methylene blue in a microwave and kept at 45 °C until use.

4.4. Add a drop of 1.5% low-melt agarose (maintained as liquid in a 45 °C heat block) to the larvae and using a 800 µm mounting capillary, and draw the larvae up head first. If positioning is not accurate the larvae can be expelled from the agarose and mounted again. Leave the capillary to cool before inserting into the lightsheet chamber.

NOTE: Alternatively, a confocal microscope could be used for this procedure. For this, larvae should be mounted laterally in agarose on a glass bottom dish.

4.5. Acquire z-stack images of the head between the eye lenses (~300 µm) and process to maximum intensity projection image.

4.5.1. Analyze brain region from images collected for total number of fluorescent cells and total intensity fluorescence[20].

4.6. Create a time lapse video of multiple projection composites over 18–24 h to track leukocyte mobility and interaction with dying cells.

NOTE: If long term live imaging is performed, only one larva is mounted in the capillary.

4.7. When imaging is completed expel the larvae from the mounting capillary into a lethal overdose of 4% MS222 to euthanize.

5. Day 3: Selecting larvae for motility assay at 72 hpf

5.1. Anaesthetize larvae in the petri dishes with 0.02% MS222.

5.2 Randomly select n = 24 larvae for motility assay and transfer into fresh E3 media and allow animals to recover from anesthetic.

NOTE: Anesthetic at this point removes selection bias for slow swimmers that are easy to catch.

6. Day 3-5: Assaying locomotion at 72, 96 and 120 hpf

6.1. Transfer larvae selected at 72 hpf into E3 medium without methylene blue.

NOTE: For assaying at 3 dpf allow the larvae ample time to recover from anesthetic (>1 h).

6.2. Plate one larva in 1 mL per well of a 24-well plate using a pipette.

NOTE: Cut the end of the pipette tip to avoid damaging the larvae. Plate size can be changed according to experimental design.

6.3. Load plates into the camera chamber and assay motion for 10 min using a white light startle routine to increase spontaneous swimming.

NOTE: Swimming behavior was tracked using a camera chamber and tracking software (see the **Table of Materials**).

6.4. Repeat experiment with the same larvae at 96 and 120 hpf. Move larvae from individual housing in assay plate to a Petri dish and incubate at 28 °C in between assays.

6.5. At assay completion, euthanize larvae in a lethal overdose of 4% MS222.

REPRESENTATIVE RESULTS:

Assessment of brain cell death using transgenic *ubiq:*secAnnexinV-mVenus results in clear definitive clusters of dying cells in ICH+ larvae in both ATV and bbh models that are absent in all ICH- larvae (**Figure 3**). Clusters recede before 96 hpf. Through image analysis, bleeding is associated with a significant two-fold increase in total intensity of fluorescence signal in the brain, indicating marked cell death.

A neuroinflammatory response is identified in ICH+ larvae by significantly increased numbers of *mpeg1* positive macrophage cells in the brain. The number of total *mpo* positive neutrophil cells also increased however this did not reach statistical significance (**Figure 4**). The morphology of the *mpeg1* positive macrophages can also be seen to change in ICH+ larvae as the cells adopt an active, rounded, amoeboid shape. These activated rounded cells can also be monitored over time to show an increased phagocytic response of the *ubiq:*secAnnexinV-mVenus expressing dying cells in ICH+ larvae (**Figure 5**). *mpeg1* positive macrophages exhibiting ramified processes were categorized as inactive.

Brain hemorrhage is associated with a significant decrease in motility at 72 and 96 hpf in comparison to ICH- sibling controls in both bbh and ATV models (**Figure 6**). Motility at 120 hpf recovers to near baseline levels. There are often differences in baseline motility between egg clutches and strains and so comparison should be made to ICH- controls every time.

FIGURE AND TABLE LEGENDS:

Figure 1: Graphic of experimental timeline to characterize brain injury, locomotor and neuroinflammatory outcomes. ICH, intracerebral hemorrhage; bbh, bubblehead. Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

Figure 2: ICH+ brain hemorrhage phenotypes. Examples of larval ICH phenotypes maintained on a transgenic gata1:DsRed reporter nacre background observed with a brightfield stereomicroscope (top panels) and fluorescence (bottom panel) at ~48 h post-fertilization. No hemorrhages were observed in ICH-larvae (left panels). A distinct accumulation of red blood cells in the forebrain and hindbrain (arrows) were observed in ICH+ larvae (right panels). Scale bars represent 250 µm. Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

Figure 3: Intracerebral hemorrhage (ICH) in zebrafish larvae results in a quantifiable brain injury. (**A**) Representative images of the brain injury phenotype in ICH+ larvae (right panels), in comparison to ICH- siblings (left panels), at 72 hpf. Brightfield images (bottom panels, scale bar = 250 µm) demonstrate the presence of brain bleeds (arrows) in ICH+ larvae. Fluorescent microscopy was performed to visualize cell death in the *ubiq:*secAnnexinV-mVenus reporter line (top panels, scale bar = 100 µm). Clusters of dying cells were observed in peri-hematomal regions. Images were cropped to brain-only regions and analyzed for total green fluorescence intensity in round particles larger than 30 pixels in diameter (white line) using the macro in **Supplementary File 1**. (**B**) Quantification of fluorescence signal in the brains of untreated, ICH- and ICH+ larvae obtained through the ATV model (n = 12 per group; 3 independent replicates) at 72 hpf. Significant differences were observed when comparing ICH+ with untreated (**p = 0.004) and with ICH- (*p = 0.03) siblings. (**C**) Quantification of fluorescence signal as a read out for annexinV binding in the brains of ICH- and ICH+ larvae obtained through the bubblehead (bbh) model (n = 12 per group; 2 independent replicates) at 72 hpf. Graphs show SD from the mean. A significant difference in mVenus fluorescence was observed between ICH+ and ICH- age-matched siblings (**p = 0.002). Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

Figure 4: Intracerebral hemorrhage (ICH) initiates an innate cellular immune response in the zebrafish larval brain. Numbers of leukocytes quantified within the brain regions previously described for mpo:GFP;mpeg1:dsRed double transgenic larvae (n = 8 per group; 2 independent replicates) at 72 hpf reveals a significant increase in macrophages (*p = 0.01), but not neutrophils (p = 0.5), in response to ICH. Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

Figure 5: Activated macrophage cells show a phagocytic response to the brain lesion. (**A**) Representative time-lapse stills[20] showing a ramified patrolling macrophage migrating towards an annexinV positive cell (i – vi). Stills are obtained from a series of images taken of the whole brain using a 20x objective. Scale bar represents 50 µm. The macrophage acquired an amoeboid morphology (v) before phagocytosing the annexinV-positive cell (vi, vii). After phagocytosis the macrophage resumes a ramified morphology and migrates away and the annexinV-positive cell can no longer be seen (viii). Ramified macrophage (#), annexinV positive cell (arrow), amoeboid macrophage (*) are indicated. (**B**) Representative images of *mpeg1*-positive cells in the ICH- and ICH+ larval brain exhibiting amoeboid and ramified morphologies. Scale bars represent 50 µm. (**C**) An increased proportion of amoeboid (phagocytic) and decreased proportion of ramified (inactive) macrophages was observed in ICH+ brains in comparison to ICH- siblings. Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

Figure 6: ICH-induced brain injury results in a quantifiable locomotor deficit in zebrafish larvae. (**A**) Representative examples of the swimming tracks in ICH- and ICH+ bbh larvae at 72, 96 and 120 hpf. (**B**) ICH+ larvae exhibited a significant decrease in the cumulative time spent mobile during the 10 min recording period at both 72 and 96 hpf. Significance was lost at the 120 hpf time point potentially alluding to recovery from brain injury (n = 24 larvae per group; 3 independent replicates; ****p = 0.00006; **p = 0.003; ns: p = 0.08). (**C**) Quantification of cumulative time spent moving in untreated and ATV-treated ICH- and ICH+ larvae at 120 hpf. ICH+ larvae exhibited a significant decrease in the cumulative time spent mobile during the 10 min recording period. Three technical replicates (n = 24 larvae per group) were used to calculate SD from the mean (***p = 0.00004, **p = 0.0003). Figure has been reproduced from Crilly et al.[20] with permission under a Creative Commons license.

DISCUSSION:

This study shows that ICH in zebrafish larvae induces a brain injury response that recapitulates key aspects of the human condition that can be systematically assayed and quantified. Zebrafish offer a consistent and reproducible model of spontaneous ICH which will assist with future drug intervention studies focused on targeting blood-induced brain injury, rather than preventing vessel rupture[17,28]. Indeed, given the rapid nature of disease onset akin to the clinical situation, such an approach offers exciting prospects for successful translation in the future.

Some limitations are associated with the use of zebrafish larvae, such as the use of a developing system and taxonomic rank, however the practical and scientific advantages of this model must be considered to offer new insights into ICH. No surgery is required to initiate a haemorrhage or to monitor cellular processes over extended periods of time after injury. High fecundity of zebrafish pairings generate easily accessible and large sample sizes, and due to the fast development of the larvae the experimental timeline is significantly reduced compared to rodent studies[29,30].

Currently these models are fit to use for elucidating the immediate pathological and immunological response to spontaneous ICH in the brain of live intact animals. Potentially, this

model can be adapted for medium-high throughput drug screens for ICH therapies, whether preventative or recovery promoting. As such, the post-ICH pathologies presented in this study represent an alternative, complementary platform for pre-clinical ICH research.

ACKNOWLEDGMENTS:

We would like to thank Dr David Spiller and the University of Manchester Systems Microscopy Core Facility for use of the equipment, Prof. Richard Baines for the use of DanioVision and Dr. Jack Rivers-Auty for statistical consultation. The bbh line was kindly shared by Nicole Munsie from Dr. Sarah Child's lab at the University of Calgary. We also thank Prof. Stephen Renshaw, Dr. Adam Hurlstone, Dr. Andrew Badrock and Dr. Helen Young for fish lines and equipment.

This study was supported by the NC3Rs (NC/N002598/1), Stroke Association (TSA LECT 2017/02), ERA-NET NEURON (MR/M501803/1) and The British Heart Foundation (FS/15/67/32038). We are also particularly thankful to The Natalie Kate Moss Trust and the University of Manchester Faculty of Biology, Medicine and Health for their continued financial support.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 An, S. J., Kim, T. J., Yoon, B.-W. Epidemiology, risk factors, and clinical features of intracerebral hemorrhage: an update. *Journal of stroke.* **19** (1), 3 (2017).

2 WHO. *The top 10 causes of death*, <http://www.who.int/mediacentre/factsheets/fs310/en/> (2017).

3 Casals, J. B. et al. The use of animal models for stroke research: a review. *Comparative medicine.* **61** (4), 305-313 (2011).

4 Kellner, C. P., Connolly, E. S. Neuroprotective Strategies for Intracerebral Hemorrhage Trials and Translation. *Stroke.* **41** (10 suppl 1), S99-S102 (2010).

5 Kirkman, M. A., Allan, S. M., Parry-Jones, A. R. Experimental intracerebral hemorrhage: avoiding pitfalls in translational research. *Journal of Cerebral Blood Flow and Metabolism.* **31** (11), 2135-2151 (2011).

6 ASPA. (1986 amendments 2012).

7 Butler, M. G., Gore, A. V., Weinstein, B. M. in *Methods in cell biology* Vol. 105 137-161 (Elsevier, 2011).

8 Walcott, B. P., Peterson, R. T. Zebrafish models of cerebrovascular disease. *Journal of Cerebral Blood Flow & Metabolism.* **34** (4), 571-577 (2014).

9 Liu, C. et al. Macrophages mediate the repair of brain vascular rupture through direct physical adhesion and mechanical traction. *Immunity.* **44** (5), 1162-1176 (2016).

10 Kasher, P. R. et al. Characterization of samhd1 morphant zebrafish recapitulates features of the human type I interferonopathy Aicardi-Goutieres syndrome. *The Journal of Immunology.* **194** (6), 2819-2825 (2015).

11 Wen, J. et al. Mutation of rnf213a by TALEN causes abnormal angiogenesis and circulation defects in zebrafish. *Brain research.* **1644** 70-78 (2016).

12 Eisa-Beygi, S., Rezaei, M. Etiology of intracerebral hemorrhage (ICH): novel insights from Zebrafish embryos. *International Journal of Developmental Biology.* **60** (4-5-6), 119-126 (2016).
13 Mracsko, E., Veltkamp, R. Neuroinflammation after intracerebral hemorrhage. *Frontiers in cellular neuroscience.* **8** 388 (2014).
14 Eisa-Beygi, S., Hatch, G., Noble, S., Ekker, M., Moon, T. W. The 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway regulates developmental cerebral-vascular stability via prenylation-dependent signalling pathway. *Developmental biology.* **373** (2), 258-266 (2013).
15 Shen, M. et al. Discovery of Rho-kinase inhibitors by docking-based virtual screening. *Molecular BioSystems.* **9** (6), 1511-1521 (2013).
16 Huang, B. et al. Tanshinone I prevents atorvastatin-induced cerebral hemorrhage in zebrafish and stabilizes endothelial cell–cell adhesion by inhibiting VE-cadherin internalization and actin-myosin contractility. *Pharmacological research.* (2017).
17 Li, S. et al. Discovery of a ROCK inhibitor, FPND, which prevents cerebral hemorrhage through maintaining vascular integrity by interference with VE-cadherin. *Cell death discovery.* **3** 17051 (2017).
18 Liu, J. et al. A βPix–Pak2a signaling pathway regulates cerebral vascular stability in zebrafish. *Proceedings of the National Academy of Sciences.* **104** (35), 13990-13995 (2007).
19 ten Klooster, J. P., Jaffer, Z. M., Chernoff, J., Hordijk, P. L. Targeting and activation of Rac1 are mediated by the exchange factor β-Pix. *The Journal of cell biology.* **172** (5), 759-769 (2006).
20 Crilly, S. et al. Using zebrafish larval models to study brain injury, locomotor and neuroinflammatory outcomes following intracerebral haemorrhage. *F1000Research.* **7** (2018).
21 Westerfield, M. The zebrafish book: a guide for the laboratory use of zebrafish. *http://zfin.org/zf_info/zfbook/zfbk.html.* (2000).
22 Ellett, F., Pase, L., Hayman, J. W., Andrianopoulos, A., Lieschke, G. J. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. *Blood.* **117** (4), e49-e56 (2011).
23 Renshaw, S. A. et al. A transgenic zebrafish model of neutrophilic inflammation. *Blood.* **108** (13), 3976-3978 (2006).
24 Traver, D. et al. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nature immunology.* **4** (12), 1238 (2003).
25 Morsch, M. et al. In vivo characterization of microglial engulfment of dying neurons in the zebrafish spinal cord. *Frontiers in cellular neuroscience.* **9** (2015).
26 Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., Schilling, T. F. Stages of embryonic development of the zebrafish. *Developmental dynamics.* **203** (3), 253-310 (1995).
27 Liang, J. O. *Zebrafish in the Classroom*, <http://www.zfic.org> (2011).
28 Yang, R. et al. Miconazole protects blood vessels from MMP9-dependent rupture and hemorrhage. *Disease Models & Mechanisms.* **10** (3), 337-348 (2017).
29 Andaluz, N., Zuccarello, M., Wagner, K. R. Experimental animal models of intracerebral hemorrhage. *Neurosurgery Clinics of North America.* **13** (3), 385-393 (2002).
30 Rosenberg, G. A., Mun-Bryce, S., Wesley, M., Kornfeld, M. Collagenase-induced intracerebral hemorrhage in rats. *Stroke.* **21** (5), 801-807 (1990).

Figure 1

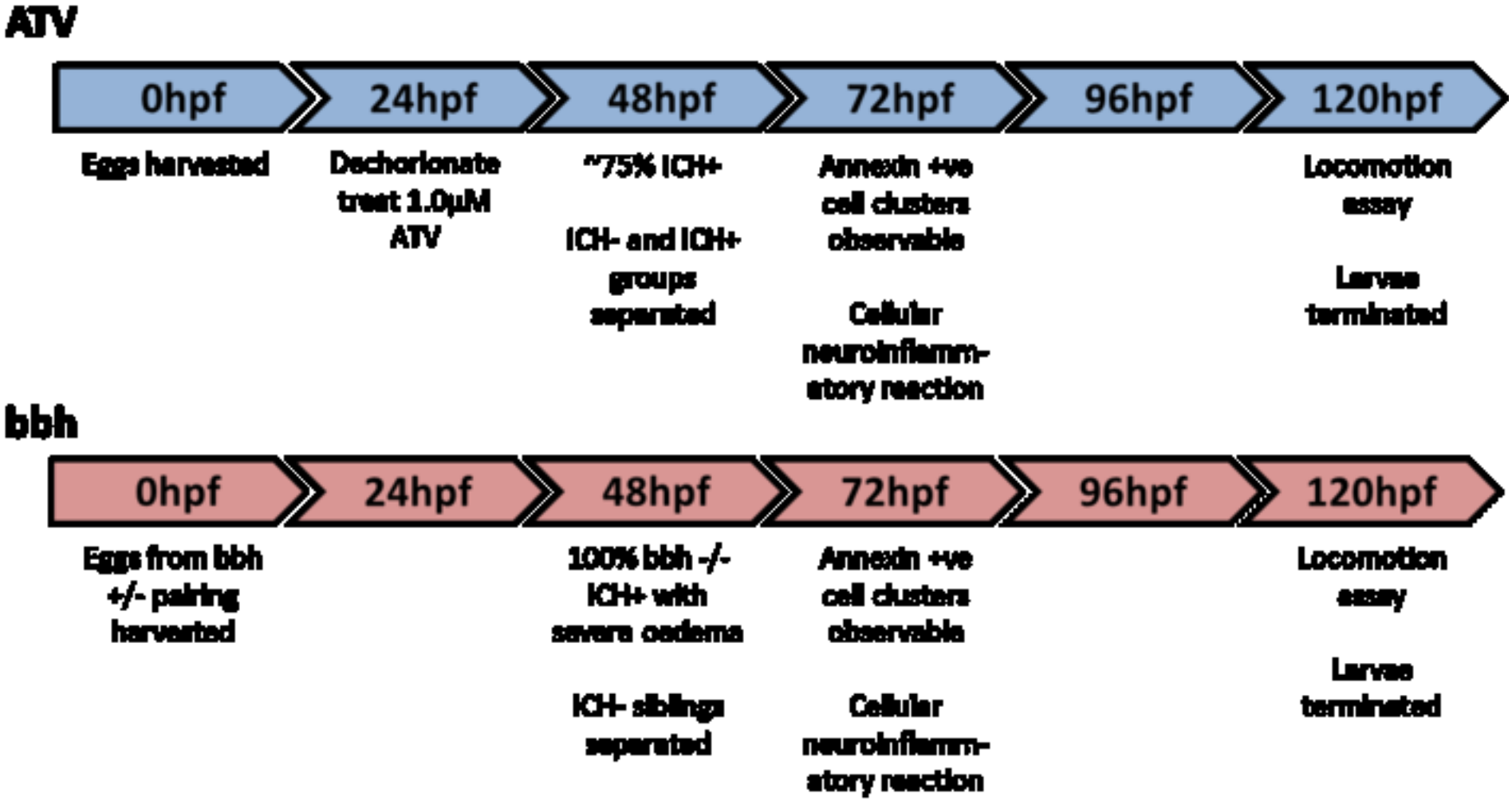


Figure 2

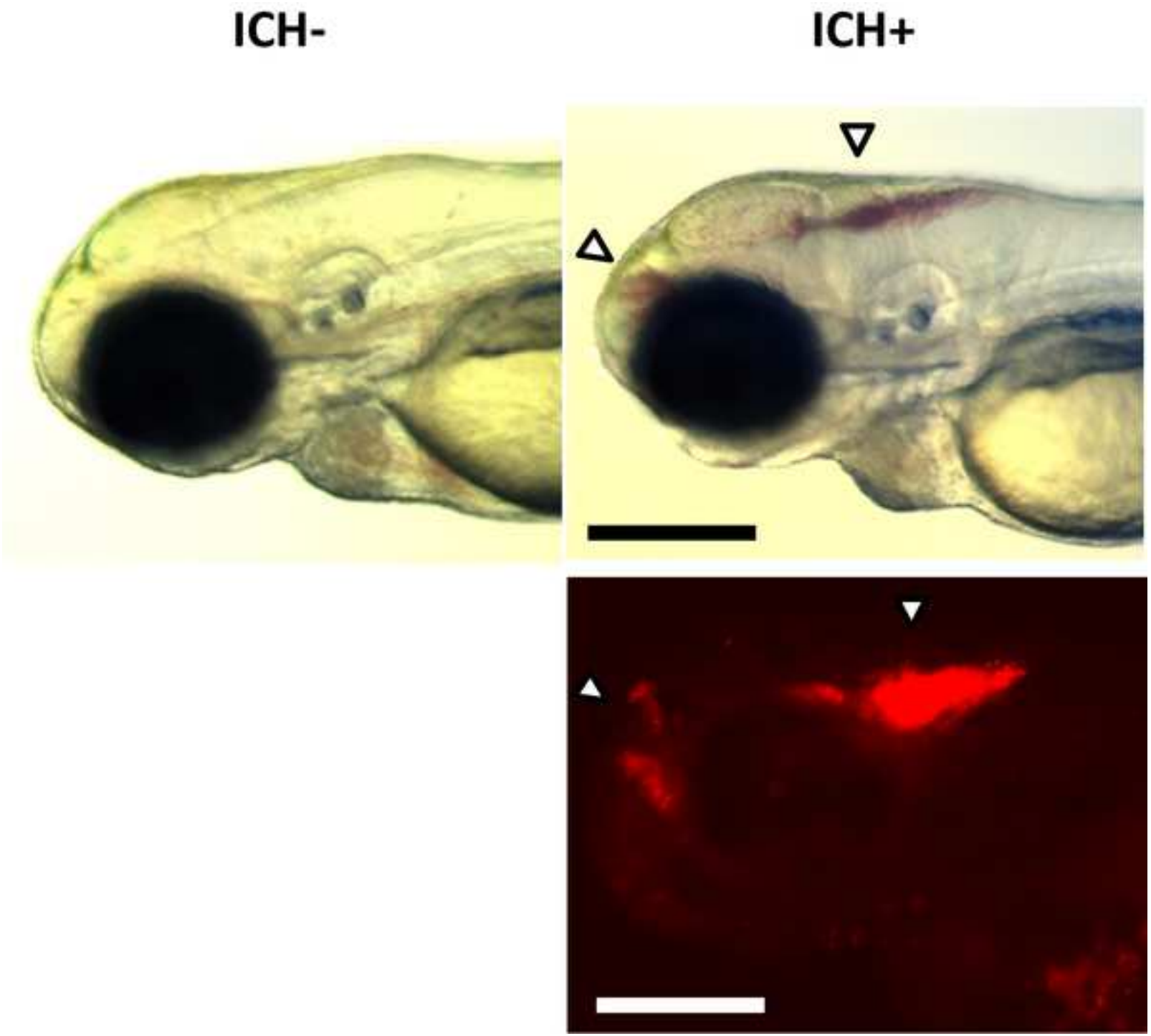


Figure 3

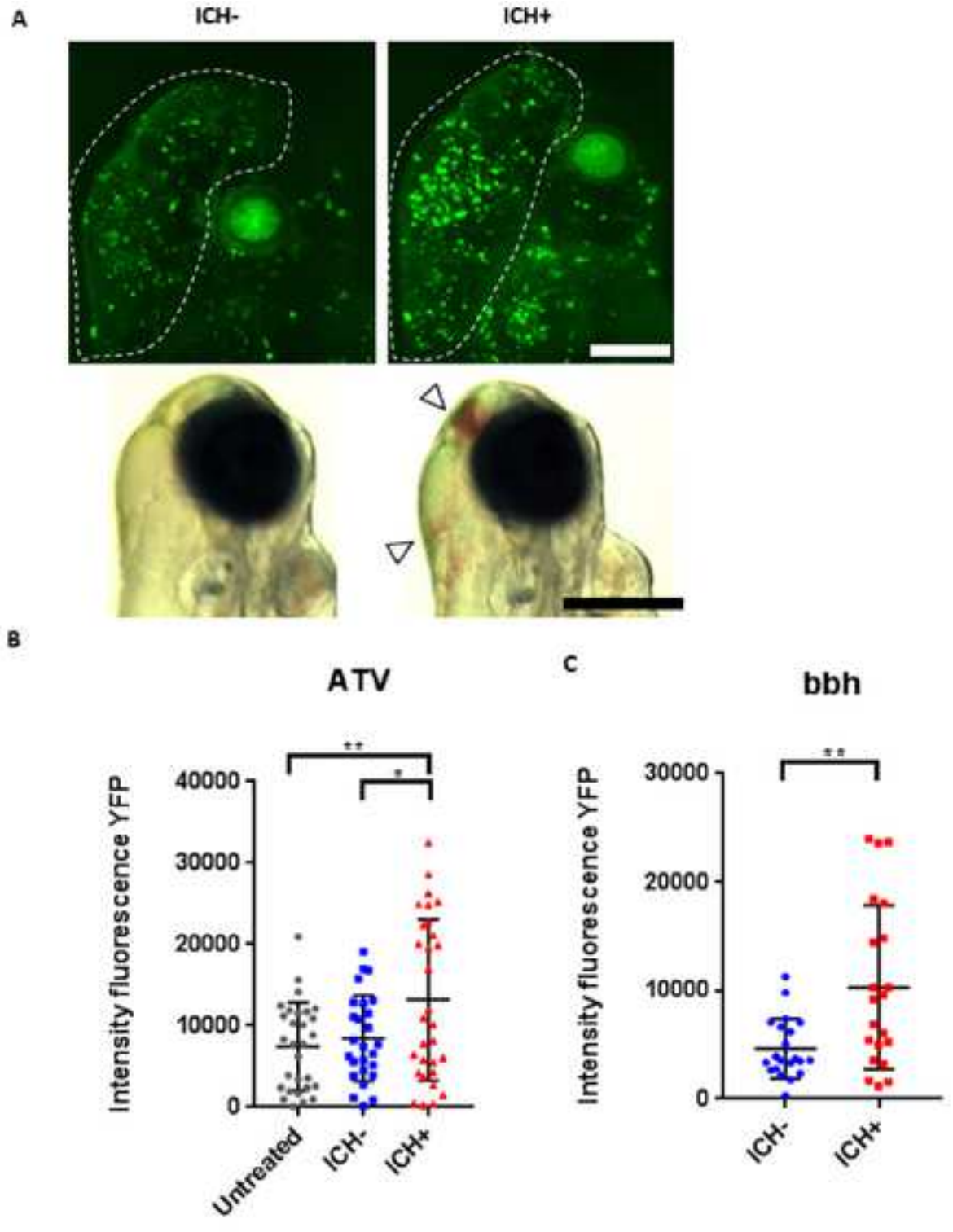


Figure 4

Click here to access/download;Figure;FIG4.TIF

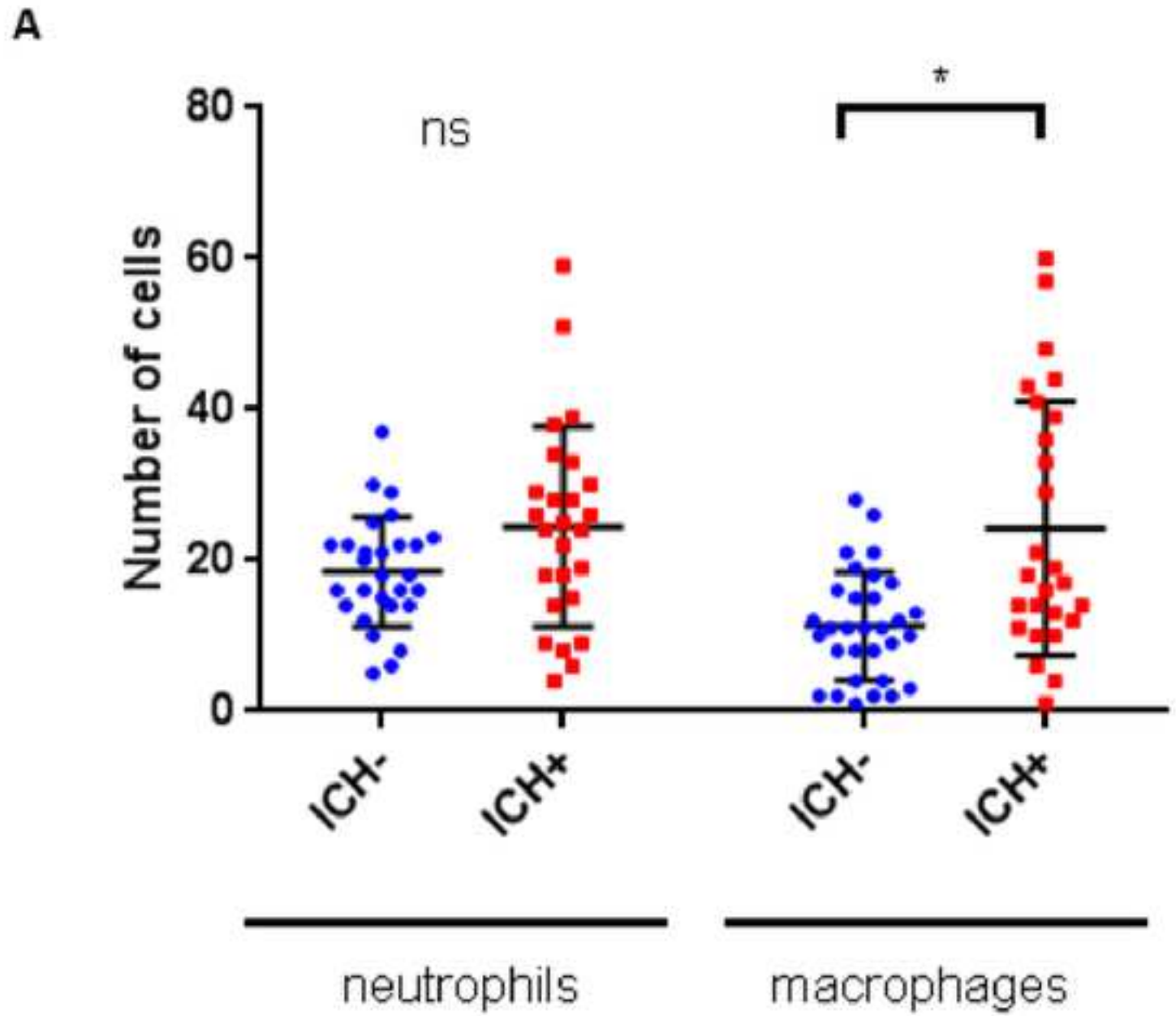


Figure 5

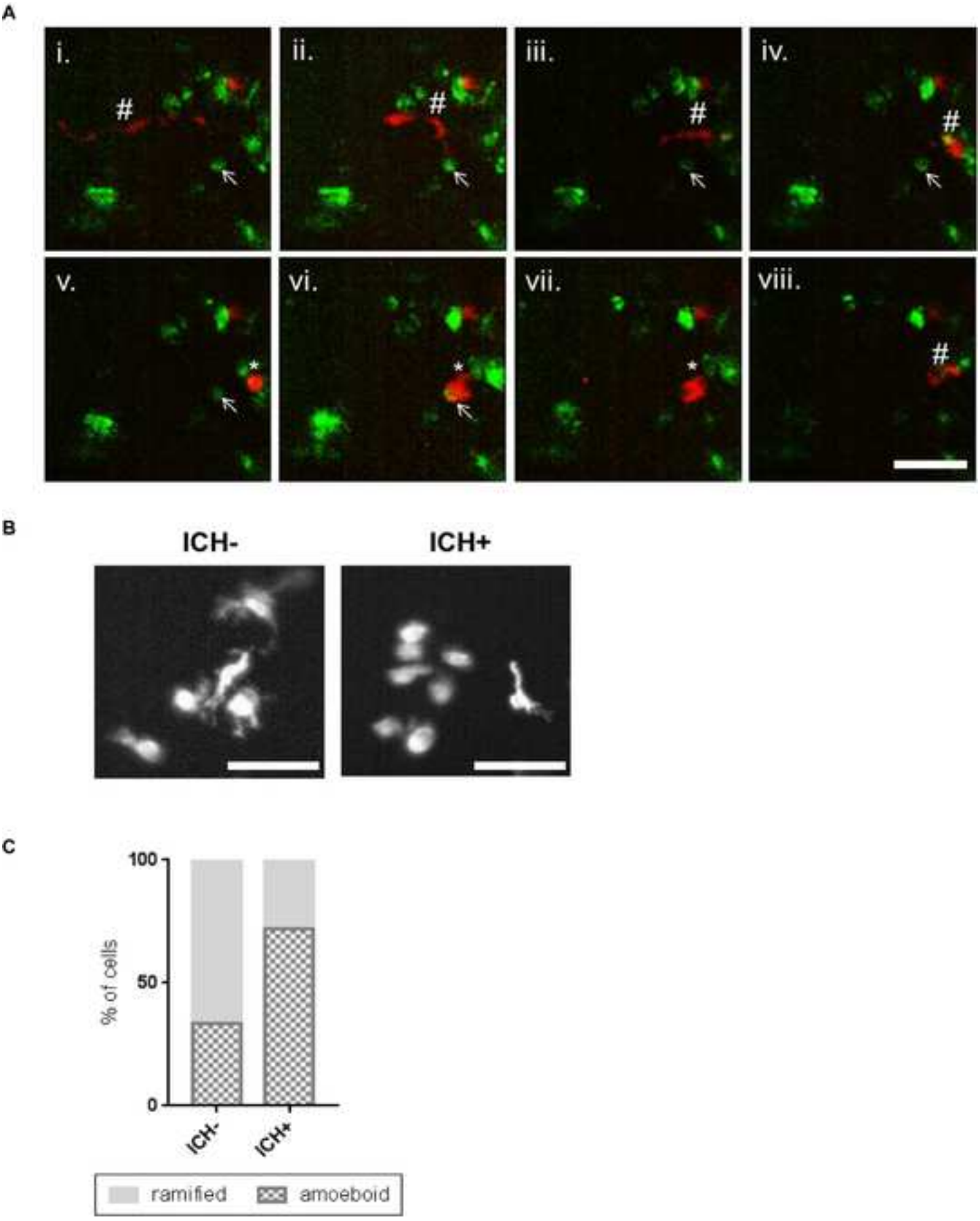
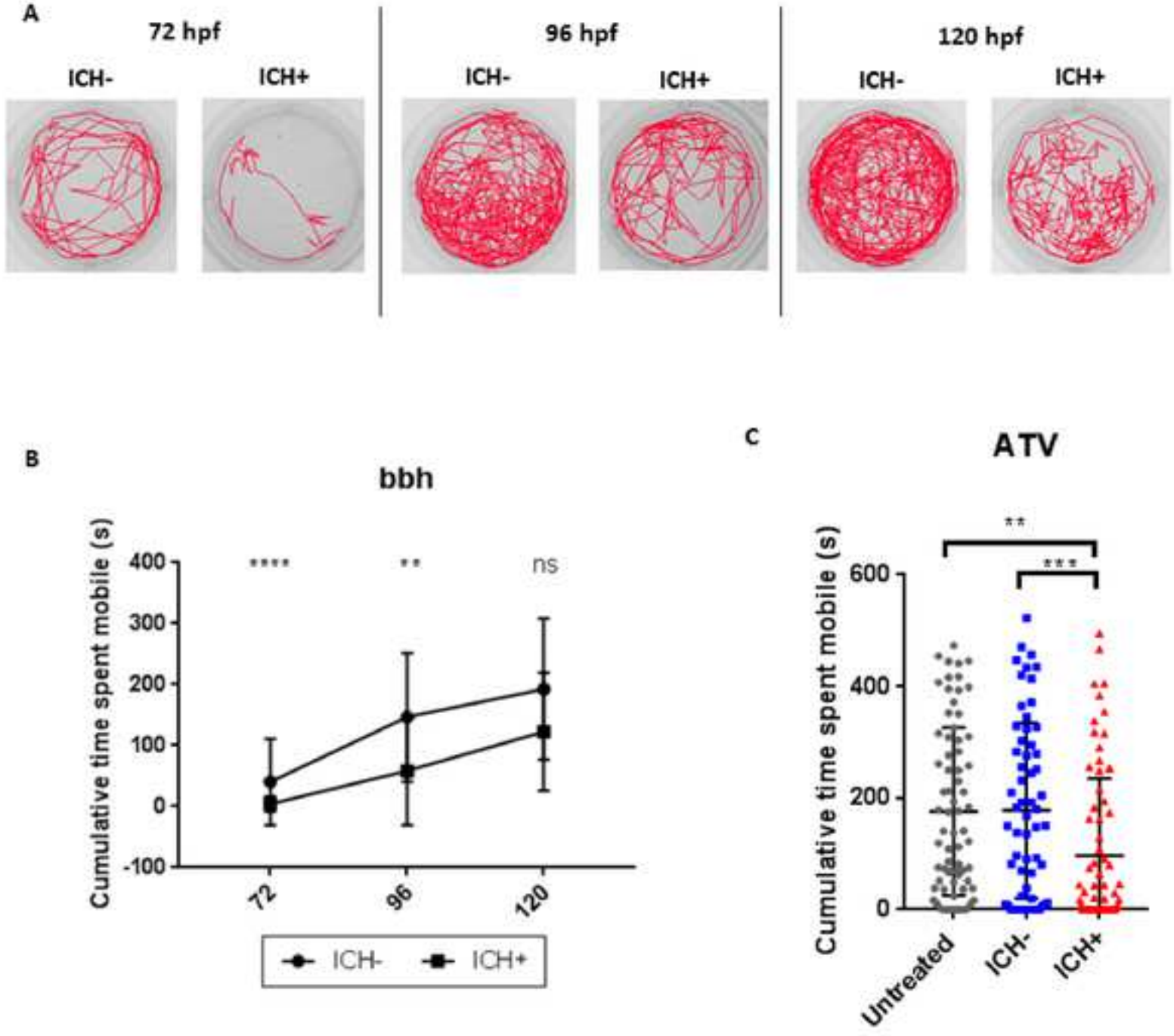


Figure 6



Name of Material/Equipment	Company	Catalog Number
24 well plates	Sigma-Aldrich	CLS3527
28 °C incubator	LMS	210
Atorvastatin	Sigma-Aldrich	PZ0001-5mg
Breeding boxes	Thoren Aquatics systems	10011
Daniovision observation chamber	Noldus	n/a
E3 medium 1x		
EthoVision XT software	Noldus	version 11
Heat block	Grant-Bio	PHMT-PSC18
Instant ocean	Instant Ocean	SS15-10
Lightsheet microscope	Zeiss	Z.1
Lightsheet microscope mounting capillary	Zeiss	402100-9320-000
Low melt agarose	Promega	V2111
Methylene Blue	Sigma-Aldrich	319112-100ML
Microscope	Leica	MZ95
Microscope	Leica	M165FC
MS222		
P1000 pipette	Gilson	F144059M
P1000 pipette tips	Starlab	S1122-1830
Pasteur pipettes	Starlab	E1414-0300
Petri dishes	Corning	101VR20
Pipetboy	Integra Biosciences	PIPETBOY
Stripette 25ml	Corning	CLS3527
Tricaine powder	Sigma-Aldrich	A5040-25G
Tris Base	Fisher BioReagents	BP152-1
Ultra fine dissection forceps	Agar scientific	AGT502
Zen software	Zeiss	version 2.3

	Comments/Description
4% Instant Ocean, 500 µL methylene blue in 1 L dH_2O	
dissection microscope fluorescent microscope 4g tricaine powder, 500 mL of dH_2O, 10 mL of 1 M Tris (pH 9). Adjust pH to ~7	

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

Title of Article:	Using zebrafish larvae to study the pathological consequences of haemorrhagic stroke
Author(s):	Siobhan Crilly, Alexandra Njegic, Adrian Parry-Jones, Stuart Allan, Paul Kasher

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution 3.0 Agreement (also known as CC-BY), the terms and conditions of which can be found at: http://creativecommons.org/licenses/by/3.0/us/legalcode ; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video - Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video - Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties**. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT - UK

discretion andwithout giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contaminationdue to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, Jo VE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	Paul Kasher
Department:	School of Biological Sciences
Institution:	The University of Manchester
Title:	Dr
Signature:	paul kasher P.K.
Date:	28/01/19

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Office 2.013
AV Hill Building
Division of Neuroscience & Experimental Psychology
School of Biological Sciences
Faculty of Biology, Medicine & Health
The University of Manchester
Oxford Road
Manchester
M13 9PT
United Kingdom

From: Paul Kasher, PhD
Position: Stroke Association HRH The Princess Margaret Lecturer
Tel: +44 (0)161 306 8059
Email: paul.kasher@manchester.ac.uk

28th February 2019

Dear Dr Dsouza,

Re: JoVE59716R1 - Using zebrafish larvae to study the pathological consequences of haemorrhagic stroke.

We would like to thank you for your email on 22nd February, and to the reviewers for their assessment of our paper. Below, I address each of the editorial and reviewer comments.

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

We have proofread our paper.

2. Please use American English.

American English has now been used throughout.

3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

All figures have been modified from Crilly et al (2018) published in *F1000Research*. These figures have been clearly cited in the legends and results section. F1000Research articles are published under a CC0 1.0 licence (please see link below). As such, we are free to reuse any of the figures or content from our original article. We have also obtained written confirmation of this agreement from James Barker via email (Senior Assistant Editor at F1000 Research). This email can be forwarded to *JoVE* upon request.

https://f1000research.com/about/legal/publicationtermsandconditions

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We have rephrased the short abstract.

5. Figure 2/3/5: Please include and define scale bars.

Scale bars have now been included in the above figures and defined within the corresponding figure legends.

6. Please define the error bars for each figure.

Error bars are all s.d from the mean and this has been included in the figure legends

Reviewers' comments:

Reviewer #1:

** Are the title and abstract appropriate for this methods article?*
Yes, but the word stroke in the title implies adult and pediatric conditions. Because locomotion in this study is assessed on larvae and not adult fish, it is recommended to narrow the title to pediatric stroke-like conditions. Similarly, the abstract needs rephrasing and the some sentences need clarification - For example, By using live imaging and motility assays brain damage,…" needs to be rephrased for clarity/grammatical purposes. (MAJOR)

In our 2018 *F1000Research* paper, we discuss these points and appreciate the limitations of the zebrafish larval model in relation to, for example, studying co-morbidities associated with human ICH in older individuals. In general, ICH can be predominantly considered as a disorder associated with older age. Consequently, it is remarkable that the phenotypes observed in developing zebrafish recapitulate those associated with the aged human brain, indicating the innate injury response to blood in the brain is both evolutionarily conserved between species and analogous during development and adulthood. Just as symptoms in pediatric cases are comparable to stroke in older patients, we have shown that larvae are an adequate model of these specific disease pathologies (i.e. cell death, locomotion and neuroinflammation), regardless of patient age. As such, we politely request to leave our title as originally written. In addition, we have altered the grammar in the long abstract as requested.

** Are there any other potential applications for the method/protocol the authors could discuss?*
The method/protocol is more relevant to embryonic condition given that fish larvae were used in this study. Applications mimicking pediatric disorders are recommended for inclusion. (MAJOR)

A specific project is ongoing in my lab characterising a mutant zebrafish model of an inherited form of childhood-onset hemorrhagic stroke. However, we consider the zebrafish models described within this manuscript to be useful for studying the immediate cellular response to blood in the brain regardless of patient age, and complementary to the existing *in-vitro* and *in-vivo* models of ICH. Our future work will focus on characterising the specific recovery mechanisms occurring in zebrafish larval models of ICH and investigating whether these developmental processes (normally dormant in the aged brain) could be activated in the patient brain. However, this is not the focus of this methodological paper and we therefore politely request to refrain from including these types of application at this stage.

** Are all the materials and equipment needed listed in the table? (Please note that any basic lab materials or equipment do not need to be listed, e.g. pipettes.)*
Annexin V is missing. Transgenic lines are missing. (MINOR)

The *ubiq*:secAnnexinV-mVenus reporter line was used throughout the paper to assay cell death and is clearly specified in the protocol and results sections. All transgenic and mutant lines used in

this study are also outlined in the protocol section. These zebrafish strains are not necessarily available commercially. As such they were originally omitted from the 'Table of Materials'.

** Do you think the steps listed in the procedure would lead to the described outcome?*
Yes, it will.
** Are the steps listed in the procedure clearly explained?*
Yes, for the most part. Annexin V staining was never explained, and the difference between ramified macrophage and presumably normal macrophage was provided in the legend but needs to be explained in the text somewhere. How do you distinguish between the two types of macrophages? This was not immediately clear. Parameters used to distinguish the two cell types would help the reader. Other queries to consider - Do bbh mutants bleed beyond 48 hpf or do the exhibit spontaneous recovery? What about the heterozygotes? Do they bleed? Have you investigated the hemorrhage in bbh hets? (MAJOR)

The *ubiq*:secAnnexinV-mVenus reporter line was used throughout the paper to assay cell death and this is clearly specified in the protocol and results sections. As stated in the results section, macrophages exhibiting an amoeboid morphology were categorised as 'activated'. Macrophages with ramified processes were categorised as 'inactive'. We have added another sentence to clarify this in the results section. We can also further explain these details during the filmed protocol. The bbh mutants do not exhibit hemorrhage beyond a critical neurovascular developmental time point (36-48hpf) and exhibit normal survival and development. Indeed, the cerebral bleed has cleared by 5dpf. Breeding homozygous adults do not exhibit hemorrhages. Liu et al (2007) detailed the mutant and hemorrhage characteristics, heterozygotes do not hemorrhage and this is also detailed in Crilly et al (2018).

** Are any important steps missing from the procedure?*
Annexin V staining, and swimming track software data procurement and analysis. (MAJOR)

We have addressed the annexin V issue above – it is a reporter line, not a stain. Swimming tracking was performed using a Daniovision camera chamber and ethovision XT software. This has been added to step 6 of the protocol.

** Are appropriate controls suggested?*
Yes, for the most part. I would encourage including avoiding pigmentation during imaging by either using transparent adult fish or PTU treatment. (MINOR)

PTU treatment can cause developmental defects, therefore we avoid its use wherever possible. Furthermore, we would also like to avoid exposure to an additional chemical, considering the larvae are also exposed to ATV. This would also be inadvisable for future drug discovery protocols. Where possible, we use zebrafish strains with pigmentation gene mutations (nacre, Casper). However not all of our mutant/reporter lines have been crossed onto such backgrounds to date. We have now included fluorescent images of the bleeds (*gata*:DsRed). To avoid misinterpretation, we have also changed Figure 2 to just show representative images of the brain haemorrhage. The phenotype is identical in both ATV and bbh models.

** Are all the critical steps highlighted?*
Yes, for the most part. In some places, the explanations could be a bit more elaborate - for example, the staining and procurement of images for locomotion analysis. (MINOR)
** Is there any additional information that would be useful to include?*
The picture showing hemorrhage in bbh homozygous mutant brains (Fig. 2) could be better. (MAJOR)

To avoid any misinterpretation, we have changed Figure 2 (and accompanying legend) to show representative images of larvae with (ICH+) and without (ICH-) brain hemorrhages. Both ATV and bbh models show identical phenotypes. We have also included images of ICH obtained from the fluorescent reporter line for erythrocytes (*gata1*:DsRed).

Points to be raised/addressed in the Discussion - (MINOR/OPTIONAL)
** When does the immune system become active in the brain? This would help understand the relevance of the macrophage infiltration finding.*

In zebrafish, the innate immune system is detectable and circulating from 1 day post-fertilization (dpf). We observe significant macrophage activation and recruitment to the brain at 3dpf (i.e. 1 day post-ICH). In humans, resident brain microglia become activated rapidly following hemorrhage (hours), with peripheral macrophages infiltrating the brain at a later stage (hours- days).

** Do statins induce bleeding at later stages? Can higher doses be used?*

Exposure to statins beyond the critical neurovascular developmental time point (36-48hpf) fails to induce ICH. Atorvastatin has been reported to be used up to 10µM in zebrafish without any other developmental defects at this time point, however we have found that brain hemorrhage is an 'all-or-nothing' response. In our hands, increasing concentration correlates to a higher number of fish exhibiting hemorrhage.

** Do other statins, like simvastatin or cerivastatin, induce the same hemorrhage phenotypes?*

Yes. As does genetic inhibition of the *hmgcra* pathway.

** Do bbh mutants develop to have hemorrhage-prone brains?*

We have examined the adult brains of homozygous bbh fish and seen no evidence of recurrent hemorrhage and so believe this is the only time point at which hemorrhage will occur by these mechanisms.

** Comparisons to hemorrhage model arising from aspirin/warfarin treatment (blood thinners)? Are they different?*

We are not aware of any zebrafish model of ICH resulting from aspirin or warfarin treatment.

** What do the vessels look like in both statin-treated and bbh mutants? Do vessels recover following bleeding. Is there more angiogenesis or delayed angiogenesis in the loci of hemorrhage.*

To date, we have not closely studied vessel morphology or angiogenesis in these models.

Reviewer #2:

Major Concerns:

- Even if it is not the topic of JoVe publication, the manuscript would greatly benefit of few sentences of how atorvastatin induces brain hemorrhage. The same for Bbh. It is not developed and these data are lacking.

We have now included a brief description of the mechanism of action for both bbh and ATV models in the introduction and the appropriate references.

- In figure 2: the authors claim that there is brain hemorrhage and show it with arrows. However, I'm not fully convinced by these results. The authors should perform a red blood cells labelling to convince readers.

To avoid any misinterpretation, we have changed Figure 2 (and accompanying legend) to show representative images of larvae with (ICH+) and without (ICH-) brain hemorrhages. Both ATV and bbh models show identical phenotypes. We have also included images of ICH obtained from the fluorescent reporter line for erythrocytes (*gata1*:DsRed).

- In figure 2: the stages of development of zebrafish larvae seem different between ATV and Bbh. On the ATV and their respective controls, there are no melanocytes while, there are some for the Bbh ones. In the legend, it is written that embryo are 50hpf.

Please see comment above.

- Figure 3: How is made the quantification of Annexin V? Is it normalized according to the total area selected? Given the strong heterogeneity/dispersion of fluorescence intensity staining in IHC+ (in ATV and Bbh models), it would be interesting to know how the authors performed their quantification. Some data are provided such as "round particles bigger than 30 pixels" but this is not sufficient. Is it possible to have a positive control inducing apotosis to ascertain the fact that this line well reflects apoptosis (H202 treatment?).
I would suggest to the authors to insert the n for each group also in the figure.

All the data was generated using a macro on imageJ, to analyse the number of the particles in the specified brain region and for total fluorescence intensity. We have now included the macro as a supplementary file, that can be opened using imageJ/Fiji and used for analysis. N numbers are included in all the figure legends, there were 3 independent replicates of 12 larvae in each group (36) and 2 replicates for bbh. With regard to a positive control, annexin V is not specific for apoptosis, therefore we can only use as a reporter for general cell death. We have removed the word ‘apoptosis’ from the manuscript – this was included in error.

- Figure 4: the graph represents the number of cells counted. However, we do not have data in the legend mentioning if it is in the whole brain, or just part of the brain. If yes, which one?

Counts were made from the whole brain region described in figure 3. This has been included in the figure legends to be more specific.

- Figure 5: there are no scale bar. Could the authors precise which part of the brain has been analyzed.

Scale bars have now been included. There is also additional reference to the Crilly et al (2018) paper where the full movie has been provided.

- Figure 6: The authors should provide the total distance traveled. On how many larvae experiment A has been done (12? 24? Replicates?). Why the authors did not provide the data for 72hpf and 96 hpf in figure 6C as for Bbh?

The data for the total distance travelled looks the same and carries the same significance as the cumulative time. We believe that the cumulative time presented more interesting and relevant data as sometimes larvae can be seen to exhibit seizure-like behaviour, and do not travel much distance but are recorded as being mobile. The data from 3dpf and 4dpf , when the larvae are so small and generally immobile is very variable for the ATV model, and other repeats of the bbh model, as the high number of 0 values makes it more suitable for a non-continuous data analysis. The significant values seen at 5dpf are much less variable and more reliably consistent.

In almost all the figures, scale bars are missing. It would be also interesting to note some anatomic structures like the eyes, the forebrain, the yolk…

Scale bars are now included. Basic anatomical features are not normally highlighted in zebrafish papers, however we can highlight these in the filming protocol if necessary.

Minor Concerns:

In the long abstract:
- the sentence "by using live imaging…" seems weird for the "brain level". I would suggest to rephrase it differently.

The grammar in this sentence has now been changed.

- the sentence "brain haemorrage in humans are conserved..." It would be appropriate to mention for what criteria.

We think it is clear that this sentence is referring to the pathological consequences of brain hemorrhage that have been specifically assayed within this paper.

Sometimes Bbh / BBh. IT is not homogenous

This issue has been corrected.

We trust we have addressed all of the editorial and reviewer comments and very much hope that you feel able to reach a positive decision regarding our manuscript.

Yours sincerely,

Paul Kasher