Journal of Visualized Experiments

A method for rapid evaluation of toxicity of chemical compounds using zebrafish embryos

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59315R2
Full Title:	A method for rapid evaluation of toxicity of chemical compounds using zebrafish embryos
Keywords:	Key Words:Zebrafish embryos, toxicity screening, in vivo toxicity, developmental toxicity, anticancer agents, phenotypic defects, pre-clinical drug development
Corresponding Author:	Ashok Aspatwar Faculty of Medicine and Health Technology Tampere, Pirkanmaa FINLAND
Corresponding Author's Institution:	Faculty of Medicine and Health Technology
Corresponding Author E-Mail:	ashok.aspatwar@tuni.fi;ashok.aspatwar@tuni.fi
Order of Authors:	Ashok Aspatwar
	Milka M Hammaren
	Mataleena Parikka
	Seppo Parkkila
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Tampere, Finland

TITLE:

Rapid Evaluation of Toxicity of Chemical Compounds using Zebrafish Embryos

Authors and affiliations:

Ashok Aspatwar[1], Milka Marjut Hammaren[1], Mataleena Parikka[1,2] Seppo Parkkila[1, 3]

[1]Faculty of Medicine and Health Technology, Tampere University, Tampere, Finland.
[2]Oral and Maxillofacial Unit, Tampere University Hospital, Tampere, Finland
[3]Fimlab Ltd, Tampere University Hospital, Finland

Email Addresses of Co-Author:

Milka Marjut Hammaren (milka.hammaren@tuni.fi)
Mataleena Parikka (mataleena.parikka@tuni.fi)
Seppo Parkkila (seppo.parkkila@tuni.fi)

Corresponding Author:

Ashok Aspatwar (ashok.aspatwar@tuni.fi)

KEYWORDS:

Zebrafish embryos, toxicity screening, in vivo toxicity, developmental toxicity, anticancer agents, phenotypic defects, pre-clinical drug development

SUMMARY:

Zebrafish embryos are used for evaluating the toxicity of chemical compounds. They develop externally and are sensitive to chemicals, allowing detection of subtle phenotypic changes. The experiment only requires a small amount of compound, which is directly added to the plate containing embryos, making the testing system efficient and cost-effective.

ABSTRACT:

The zebrafish is a widely used vertebrate model organism for the disease and phenotype-based drug discovery. The zebrafish generates many offspring, has transparent embryos and rapid external development. Zebrafish embryos can, therefore, also be used for the rapid evaluation of toxicity of the drugs that are precious and available in small quantities. In the present article, a method for the efficient screening of the toxicity of chemical compounds using 1-5-day post fertilization embryos is described. The embryos are monitored by stereomicroscope to investigate the phenotypic defects caused by the exposure to different concentrations of compounds. Half-maximal lethal concentrations (LC_{50}) of the compounds are also determined. The present study required 3-6 mg of an inhibitor compound, and the whole experiment takes about 8-10 h to be completed by an individual in a laboratory having basic facilities. The current protocol is suitable for testing any compound to identify intolerable toxic or off-target effects of the compound in the early phase of drug discovery and to detect subtle toxic effects that may be missed in the cell culture or other animal models. The method reduces procedural delays and costs of drug development.

INTRODUCTION:

Drug development is an expensive process. Before a single chemical compound is approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) several thousand compounds are screened at a cost of over one billion dollars[1]. During the preclinical development, the largest part of this cost is required for the animal testing[2]. To limit the costs, researchers in the field of drug development need alternative models for the safety screening of chemical compounds[3]. Therefore, in the early phase of the drug development, it would be very beneficial to use a method that can rapidly evaluate the safety and toxicity of the compounds in a suitable model. There are several protocols that have been used for the toxicity screening of chemical compounds involving animal and cell culture models but there is not a single protocol that is validated and is in common use[4-5]. Existing protocols using zebrafish vary in length and have been used by individual researchers who evaluated the toxicity as per their convenience requirement[6-12].

In the recent past, the zebrafish has emerged as a convenient model for the evaluation of the toxicity of chemical compounds during embryonic development[6-7]. The zebrafish has many in-built advantages for the evaluation of chemical compounds[13]. Even large-scale experiments are amenable, as a zebrafish female can lay batches of 200-300 eggs, which develop rapidly ex- vivo, do not need external feeding for up to a week and are transparent. The compounds can be added directly into the water, where they can (depending on the nature of the compound) diffuse through the chorion, and after hatching, through the skin, gills and mouth of larvae. The experiments do not require copious amounts of chemical compounds[14] due to the small size of the embryo. Developing zebrafish embryos express most of the proteins required to achieve the normal developmental outcome. Therefore, a zebrafish embryo is a sensitive model to assess whether a potential drug can disturb the function of a protein or signaling molecule that is developmentally significant. The organs of the zebrafish become functional between 2-5 dpf[15], and compounds that are toxic during this sensitive period of embryonic development induce phenotypic defects in zebrafish larvae. These phenotypic changes can be readily detected using a simple microscope without invasive techniques[11]. Zebrafish embryos are widely used in toxicological research due to their much greater biological complexity compared to in vitro drug screening using cell culture models[16-17]. As a vertebrate, the genetic and physiologic makeup of zebrafish is comparable to humans and hence toxicities of chemical compounds are similar between zebrafish and humans[8,18-22]. Zebrafish is, thus, a valuable tool in the early phase of drug discovery for the evaluation of toxicity and safety of the chemical compounds.

In the present article, we provide a detailed description of the method used for evaluating the safety and toxicity of carbonic anhydrase (CA) inhibitor compounds using 1-5-day post fertilization (dpf) zebrafish embryos by a single researcher. The protocol involves exposing zebrafish embryos to different concentrations of chemical inhibitor compounds and studying the mortality and phenotypic changes during the embryonic development. At the end of the exposure to the chemical compounds, the LC_{50} dose of the chemical is determined. The method allows an individual to carry out efficient screening of 1-5 test compounds and takes about 8-10 h depending on the experience of the person with the method (**Figure 1**). Each of the steps required to assess the toxicity of the compounds is outlined in **Figure 2.** The evaluation of toxicity

of CA inhibitors requires 8 days, and includes setting up of mating pairs (day 1); collection of embryos from breeding tanks, cleaning and transferring them to 28.5 °C incubator (day 2); distribution of the embryos into the wells of a 24-well plate and addition of diluted CA inhibitor compounds (day 3); phenotypic analysis and imaging of larvae (day 4-8), and determination of LC_{50} dose (day8). This method is rapid and efficient, requires a small amount of the chemical compound and only basic facilities of the laboratory.

PROTOCOL:

The zebrafish core facility at Tampere University has an establishment authorization granted by the National Animal Experiment Board (ESAVI/7975/04.10.05/2016). All the experiments using zebrafish embryos were performed according to the Provincial Government of Eastern Finland, Social and Health Department of Tampere Regional Service Unit protocol # LSLH-2007-7254/Ym-23.

1. Setting up of overnight zebrafish mating tanks

1.1 Place 2-5 adult male zebrafish and 3-5 adult female zebrafish into mating tanks overnight. Induce the breeding in the morning by turning the light on.

1.2 Set up several crosses to obtain enough embryos for assessing the toxicity of more than two chemical compounds. For the evaluation of toxicity, each concentration needs a minimum of 20 embryos[23].

1.3. To avoid handling stress to the animals, allow the animals to rest for 2 weeks before using the same individuals for breeding.

2. Collection of embryos and preparing plates for exposure to the chemical compounds

2.1 Collect the embryos, the next day before noon, using a fine-mesh strainer and transfer them onto a Petri dish containing E3 embryo medium [5.0 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 0.1% w/v Methylene Blue].

2.2 Remove debris using a plastic Pasteur pipette (e.g., food and solid waste). Examine each batch of embryos under the stereomicroscope to remove the unfertilized/dead embryos (identified by their opaque appearance).

2.3 Keep the embryos at 28.5 °C in an incubator. Examine the embryos, the next morning, under a stereomicroscope and remove any unhealthy or dead embryos. Also, replace the old E3 medium with fresh E3 medium.

NOTE: Zebrafish embryos are always maintained at 28.5 °C under laboratory conditions.

2.4 Carefully transfer 1 embryo into each well of a 24-well plate containing enough E3 medium to cover the embryos.

3. Preparations of the stock solution of chemical compounds and distribution of diluted compound into the wells

3.1 Take out the vials containing inhibitor compounds stored at 4 °C.

NOTE: Depending on the properties of the compound, these are stored at different temperatures.

3.2 Weigh the compound(s) using an analytical balance that can weigh a few milligrams (mg) of the compound accurately.

3.3. Prepare at least 250 µL (100 mM) of stock solution for each compound in an appropriate solvent (e.g., E3 water or Dimethyl sulfoxide (DMSO), based on the solubility properties of the compounds.

NOTE: The above steps can be done a day before the start of the experiment at a convenient time and stored at 4 °C).

3.4. Make serial dilutions of the stock solutions (e. g., 10 µM, 20 µM, 50 µM, 100 µM, 150 µM, 300 µM and 500 µM) using E3 water in 15 mL centrifuge tubes.

NOTE: The concentrations and number of serial dilutions vary from one compound to another compound depending on their toxicity levels.

3.5. From the 24 well plate containing embryos, remove E3 water from the wells using a Pasteur pipette and a 1 mL pipette (containing 1 dpf embryos) one row at a time.

3.6. Distribute 1 mL of each diluent in each well (starting from lower and moving to higher concentration) into the wells of 24-well plate.

3.7. Set up a control group from the same batch of embryos and add the corresponding amount of diluent.

3.8. Label 24-well plates with the name and concentration of the compound and keep the plates at 28.5 °C in an incubator.

4. Phenotypic analysis and imaging of the embryos using a stereomicroscope

4.1. Examine the embryos under a stereomicroscope for parameters 24 h after exposure to the chemical compounds.

4.1.1. Note the parameters such as mortality, hatching, heartbeat, utilization of yolk sack, swim bladder development, movement of the fish, pericardial edema, and shape of the body[23].

4.1.2. Take the larvae exposed to each concentration of the compound and lay them sideways in a small Petri dish containing 3% high molecular weight methyl cellulose using a metal probe.

NOTE: The 3% methyl cellulose (high molecular with) is a viscous liquid needed for embedding the fish with a required orientation for microscopic examination. For orienting the fish in this liquid, a metal probe is needed.

4.1.3. Take the images using stereomicroscope attached to a camera. Save the images in a separate folder each day till the end of the experiment.

4.1.4. Enter all the observations in a table each day either in an online table or on a printed sheet.

4.1.5. If the compounds are neurotoxic, the 4 to 5-dpf larvae may show altered swim pattern, make a record of such changes either by capturing a short (30 s to 1 min) video of the larvae exhibiting abnormal movement pattern**.**

4.1.6. After 5 days of exposure to the chemical compounds, note the concentration at which half of the embryos die for calculating the half maximal lethal concentration 50 (LC_{50}) of each chemical.

NOTE: The LC_{50} is the concentration at which 50% of the embryos die at the end of 5 days of exposure to a chemical compound. Use a minimum of 20 embryos for testing the toxicity of each concentration of a compound[23].

4.1.7. Construct a curve for mortality of embryos for all the concentration using a suitable program.

REPRESENTATIVE RESULTS:

The critical part of the evaluation of toxicity is testing different concentrations of one or multiple chemical compounds in a single experiment. In the beginning, select the compounds for evaluation of toxicity, the number of concentrations to test for each compound, and accordingly, make a chart **(Figure 3)**. We used a unique color for each compound to organize the samples **(Figure 3).** The use of solvent resistant marker and labeling at the bottom or sides of the plates is important to avoid mix-up later. If the compounds induce any phenotypic defects in the larvae exposed to different concentrations of inhibitors, the defects are recorded every 24 h over the period of 1-5 dpf (**Figure 4A,B,C,D**). The embryos treated with a known CA IX inhibitor at a concentration of 500 µM did not show any apparent phenotypic changes in the 1-5 days of exposure to the chemical compound (**Figure 4A**). **Figure 4C** and **Figure 4D** shows the embryos treated with β-CA inhibitors that induced various phenotypic defects unhatched embryos even at day 3 (arrow head), curved body structure (arrow), unutilized yolk sack and pericardial edema (arrow head) and absence of otolith sacs in the larvae at 5 days after treatment with chemical compound (arrow). In another study, the embryos treated with CA inhibitor (**Figure 4E**) shows the absence of otolith sacs and swim bladder (arrow heads). In our study, (**Figure 4C,D**)**,** we

documented phenotypic defects (fragile embryos and absence of pigmentation) even after day 1 of exposure to CA inhibitors. The phenotypic analyses showed that some of the inhibitor compounds are lethal and cannot be developed as drugs for human use (**Figure 4C,D** and **Table 1**).

The experiments identified a representative compound which induced minimal or no phenotypic changes during embryonic development and showed a high LC_{50} dose (**Figure 5**), suggesting that the compound is safe for further characterization and can be potentially developed into a drug candidate for human use[16]. Looking at still images of larvae exposed to the compound showed no phenotypic defects (**Figure 4A, B**). However, the same compound was found to be neurotoxic and induced ataxia in the larvae after 5-days of exposure to the CA inhibitor (**Figure 6**). This phenotype could only be detected by directly observing the swimming behavior of the zebrafish larvae under the microscope. These studies suggested that the neural development of zebrafish larvae is sensitive to the compound[16-17].

FIGURES AND TABLES LEGENDS

Figure 1: Time in h required for the rapid screening of chemical compounds using zebrafish embryos: In one set of experiments, a person with hands-on experience can screen about 5 chemical compounds (each compound requiring a minimum of 6 dilutions) using zebrafish embryos in 24-well plates. In total, it takes about 8-10 h from the setting up of crosses to performing LC_{50} determination over a period of 8 days.

Figure 2: Chart showing the breakdown of toxicity evaluation of chemical compounds. Toxicity screening of chemical compounds requires 8 days and can be broken down into five steps. (**Day 1**) Consists of setting up of zebrafish mating pairs in tanks. (**Day 2**) Involves collecting embryos from the mating tanks into Petri dishes followed by keeping them at a 28.5 °C incubator for overnight. (**Day 3**) Examination of embryos using a stereomicroscope and cleaning the embryos. Distribution of the embryos into 24-well plates and adding the dilutions of the test compounds. (**Day 4-8**) Phenotypic analyses of larvae for developmental defects. Swim pattern study and LC_{50} determination was performed on the last day of exposure to compounds.

Figure 3: Schematic presentation of experiments involved in toxicity evaluation. The toxicity evaluation consists of preparing tabulated chart containing information about chemical compounds, dilutions of the compounds and parameters to be assessed. Making the dilution of stock solutions to the desired concentrations in 15 mL centrifuge tubes (the dilution from one tube to be added to each row of the 24-well plate. A 24-well plate marked with a water proof marker with the name of the test compound, concentration in each row, and date of exposure. Examination and imaging of embryos by transferring the larvae onto a small Petri dish containing 3% high molecular weight methyl cellulose.

Figure 4: Example of phenotypic analysis of the larvae in the control and test compound treated groups. (**A**) Phenotypic analyses of the control group larvae not treated with any compound showed no phenotypic defect under a microscope. (**B**) Larvae treated with 500 µM CA inhibitor

(known inhibitor of carbonic anhydrase IX showed no observable phenotypic defects. (**C**, **D**) The developing larvae treated with β-CA inhibitor with concentrations of 250 µM and 125 µM respectively. The compounds induced phenotypic defects such as curved body, pericardial edema, and unutilized yolk sac (arrows and arrow heads). Panels **A** and B has been modified from Aspatwar et al[16]. The panels C, **D** and **E** show previously unpublished images, which were obtained using a different microscope.

Figure 5: Determination of lethal concentration 50 (LC_{50}) using 1-5 dpf zebrafish larvae. Toxicity assessment using zebrafish embryos allows researchers to determine the minimum lethal concentration of the chemical compound at the end of the experiment. The LC_{50} concentration of the compound (a known CA IX inhibitor) was 3.5 mM. The high LC_{50} dose allows further characterization of the compound. This figure has been modified from Aspatwar et al[16].

Figure 6: An example of swim pattern analysis in both untreated (A) and inhibitor treated (B) groups of larvae. The zebrafish larvae treated with 300 µM test inhibitor compound (a known inhibitor of human carbonic anhydrase IX) showed an abnormal (ataxic) movement pattern, suggesting that the compound induces neurotoxicity in the developing embryos. The arrowheads point to the normal curvature of the tail during swimming. This figure has been modified from Aspatwar et al[16].

Table 1: A summary of the safety and toxicity of the compounds screened. The toxicity evaluation experiment helps the researcher to reach a conclusion about the safety of the tested chemical compounds. The LC_{50} concentration allows to define safe concentration for further characterization. A researcher can decide if the compound is lethal even after 24 h of exposure of embryos to the compound under investigation. In our example, a subtle effect on swimming due to neurotoxicity is the significant information, which is helpful for setting up further experiments. Accordingly, based on the toxicity screening, we were able to use safe concentrations of the CA inhibitor for further characterization in vivo[24].

DISCUSSION:

In vitro toxicity test using cultured cells can detect survival and morphological studies of the cells providing limited information about the toxicity induced by the test compound. The advantage of toxicity screening of chemical compounds using zebrafish embryos is rapid detection of chemically induced phenotypic changes in a whole animal during embryonic development in a relevant model organism. Approximately 70% of protein-coding human genes have orthologs counterparts in the zebrafish genome[25]. Genetic pathways controlling the signal transduction and development are highly conserved between human and zebrafish[26], and, therefore, chemical compounds are likely to have similar toxic effects in humans[25].

Zebrafish are at the forefront of toxicological research and have already been extensively used to detect toxins in water samples and to study the mechanism of action of environmental toxins and their effects on the animal[27]. In this article, we show the use of developing zebrafish embryos to evaluate the toxicity of potential compounds at the early phase of drug discovery. Our rapid and multifaceted toxicity assessment is based on 1) changes in the phenotype of the organism

(hatching, otolith sacs, pericardial edema, yolk sac utilization, notochord development, heartbeat, swim bladder development, movement) during embryonic development over period of 5 days, and 2) determination of the lethal dose (LC_{50}). Reasonable hours of hands-on work (8-10 h divided over 8 days) is enough to determine the toxicity profile of a compound using this procedure. For newly synthesized compounds that are available in limited amounts, toxicity can be evaluated using a minimum of 3 mg of the compound. No special equipment is required. The procedure is, thus, a rapid and low-cost way to evaluate the toxic effects of any compound that can potentially be developed into the drug for human use.

The quality of the batch of embryos has a great impact on the outcome of the experiment. The quality of eggs (including the rate of fertilization) is not obvious immediately after collection from the breeding tanks (0-4 hpf). For obtaining only normally developing embryos for the experiments, before adding compounds, we allow the embryos to remain at 28.5 °C for 24 h after the collection from the breeding tanks. After 24 hpf, any dead or unhealthy-looking embryos are discarded. In addition to this, it is advisable to have a mock-treated group of embryos in each experiment to further control for the quality of the batch of eggs used in the experiment as well as to see the baseline mortality to accurately determine LC_{50}.

A mock-treated group is also needed to control for the toxicity of the vehicle of choice. Many chemicals are insoluble in water and DMSO is often used as the vehicle for the delivery of the test compounds. DMSO is generally well tolerated by the embryos in lower (0.1%) concentrations[28]. Sometimes, the compounds are not completely soluble even in DMSO and the solution appears cloudy making it difficult for the evaluation of the toxicity. In such cases, to get the stock solution of the compound completely soluble and clear, adding a drop of 0.1% NaOH will solve the problem. Appropriate control groups need to be set up for assessing the toxicity of the compound accurately. If other vehicles are used, their inherent toxicity might affect the experiment.

Each well of a 24-well plate contains from 1-10 embryos submerged in a total volume of 1 mL. If the test compound is available only in small amounts, it may be necessary to use up to 10 embryos per well for evaluating its toxicity. It is highly recommended that only 1 embryo per well should be used for each analysis[16,24,29]. The plate is stored at 28.5 °C incubator for 5 days. Sometimes significant evaporation is observed causing a marked change in the concentration of the compound in a given well[16]. By sealing the 24-well plates with paraffin films from all sides, placing embryos only in the middle wells and filling the other wells along the rims with water, the problems caused by evaporation can be avoided. In our earlier studies, we did not observe any evaporation of the diluents of the chemicals from 24-well plates[24,29]. Also, if the compound in question is known to be unstable under ambient temperatures, daily changes of water with fresh compound will be needed for reliable results.

The swim pattern of zebrafish larvae is analyzed after the 5-day exposure to the compound. The wells of a 24-well plate containing 5-dpf larvae are not ideal for the purpose due to the limited size of the well. Therefore, for accurate analysis of swim pattern, the larvae need to be transferred to a Petri dish containing 50 ml of E3 water and can settle for 2 min before the

analysis. In our study, only two inhibitors showed the effect on swim pattern[16] among the 52 α-CA and β-CA inhibitors screened for safety and toxicity, based on our experience this test can detect subtle changes including mild ataxic movement of the larvae.

This study demonstrated that the only limitation to the current method is physical dexterity. This concern can be overcome through repetition, as the skill of a person improves with practice. Once the expertise is achieved the evaluation of compounds using zebrafish embryos is ethical, easy, efficient and informative. We, therefore, expect that such a rapid assay using zebrafish embryos will become a popular tool for the in vivo toxicity screening in the early phase of drug development.

ACKNOWLEDGMENTS:

The work was supported by grants from Sigrid Juselius Foundation (SP, MP), Finnish Cultural Foundation (AA, MH), Academy of Finland (SP, MP), Orion Farmos Foundation (MH), Tampere Tuberculosis Foundation (SP, MH and MP) and Jane and Aatos Erkko Foundation (SP and MP). We thank our Italian and French collaborators, Prof. Supuran, and Prof. Winum, for providing carbonic anhydrase inhibitors for safety and toxicity evaluation for anti-TB and anti-cancer drug development purposes. We thank Aulikki Lehmus and Marianne Kuuslahti for the technical assistance. We also thank Leena Mäkinen and Hannaleena Piippo for their help with the zebrafish breeding and collection of embryos. We sincerely thank Harlan Barker for critical evaluation of the manuscript and insightful comments.

DISCLOSURES:

No potential conflict of interest was reported by the authors.

REFERENCES:

1. Amaouche, N., Casaert Salome, H., Collignon, O., Santos, M. R., Ziogas, C. Marketing authorization applications submitted to the European Medicines Agency by small and medium-sized enterprises: an analysis of major objections and their impact on outcomes. *Drug Discovery Today.* **23** (10), 1801-1805 (2018).
2. Garg, R.C., Bracken, W.M., Hoberman, A.M. In: Reproductive and Developmental Toxicology. In Reproductive and developmental safety evaluation of new pharmaceutical compounds, Gupta RC, Ed. Elsevier: Boston, MA, USA, 89-109 (2011).
3. Lee, H. Y., Inselman, A. L., Kanungo, J., Hansen, D. K. Alternative models in developmental toxicology. *Systems Biology in Reproductive Medicine*. **58** (1), 10-22 (2012).
4. Gao, G., Chen, L., Huang, C., Anti-cancer drug discovery: update and comparisons in yeast, Drosophila, and zebrafish. *Current Molecular Pharmacology.* **7** (1), 44-51 (2014).
5. Brown, N. A. Selection of test chemicals for the ECVAM international validation study on in vitro embryotoxicity tests. European Centre for the Validation of Alternative Methods. *Alternatives to Laboratory Animals.* **30** (2), 177-98 (2002).
6. Selderslaghs, I. W., Van Rompay, A. R., De Coen, W., Witters, H. E. Development of a screening assay to identify teratogenic and embryotoxic chemicals using the zebrafish embryo. *Reproductive Toxicology* (Elmsford, N.Y.) **28** (3), 308-320 (2009).

7. Brannen, K. C., Panzica-Kelly, J. M., Danberry, T. L., Augustine-Rauch, K. A. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. *Birth Defects Research Part B Developmental and Reproductive Toxicology.* **89** (1), 66-77 (2010).
8. Hermsen, S. A., van den Brandhof, E. J., van der Ven, L. T., Piersma, A. H. Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their *in vivo* potencies. *Toxicology in Vitro.* **25** (3), 745-753 (2011).
9. Lessman, C. A. The developing zebrafish (Danio rerio): a vertebrate model for high-throughput screening of chemical libraries. *Birth Defects Research. Part C, Embryo Today: Reviews.* **93** (3), 268-80 (2011).
10. Lantz-McPeak, S., et al. Developmental toxicity assay using high content screening of zebrafish embryos. *Journal of Applied Toxicology.* **35** (3), 261-72 (2015).
11. Truong, L., Harper, S. L., Tanguay, R. L., Evaluation of embryotoxicity using the zebrafish model. *Methods in Molecular Biology.* **691**, 271–279 (2011).
12. Rodrigues, G. C. et al. Design, synthesis, and evaluation of hydroxamic acid derivatives as promising agents for the management of Chagas disease. *Journal of Medicinal Chemistry.* **57** (2), 298-308 (2014).
13. Kanungo, J., Cuevas, E., Ali, S. F., Paule, M. G. Zebrafish model in drug safety assessment. *Current Pharmaceutical Design.* **20** (34), 5416-5429 (2014).
14. Peterson, R. T., Link, B. A., Dowling, J. E., Schreiber, S. L. Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proceedings of the National Academy of Sciences of the United States of America.* **97** (24), 12965-12969 (2000).
15. Stainier, D. Y., Fishman, M. C. The zebrafish as a model system to study cardiovascular development. *Trends in Cardiovascular Medicine*. **4** (5), 207-212 (1994).
16. Aspatwar, A. et al. Nitroimidazole-based inhibitors DTP338 and DTP348 are safe for zebrafish embryos and efficiently inhibit the activity of human CA IX in Xenopus oocytes. *Journal of Enzyme Inhibition and Medicinal Chemistry.* **33** (1), 1064-1073 (2018).
17. Rami, M. et al. Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates. *Journal of Medicinal Chemistry*. **56** (21), 8512-8520 (2013).
18. Spitsbergen, J. M., Kent, M. L. The state of the art of the zebrafish model for toxicology and toxicologic pathology research--advantages and current limitations. *Toxicologic Pathology*. **31** Suppl, 62-87 (2003).
19. Teraoka, H. et al. Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish. *Biochemical and Biophysical Research Communications.* **304** (2), 223-228 (2003).
20. Zon, L. I., Peterson, R. T. *In vivo* drug discovery in the zebrafish. *Nature Reviews Drug Discovery.* **4** (1), 35-44 (2005).
21. Hill, A. J., Teraoka, H., Heideman, W., Peterson, R. E. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences*. **86** (1), 6-19 (2005).
22. Kari, G., Rodeck, U., Dicker, A. P. Zebrafish: an emerging model system for human disease and drug discovery. *Clinical Pharmacology and Therapeutics*. **82** (1), 70-80 (2007).
23. Gourmelon, A., Delrue, N. Validation in Support of Internationally Harmonised OECD Test Guidelines for Assessing the Safety of Chemicals. *Advances in Experimental Medicine and Biology.* **856**, 9-32 (2016).

24. Aspatwar, A. et al. beta-CA-specific inhibitor dithiocarbamate Fc14-584B: a novel antimycobacterial agent with potential to treat drug-resistant tuberculosis. *Journal of Enzyme Inhibition and Medicinal Chemistry.* **32** (1), 832-840 (2017).
25. Kazokaite, J., Aspatwar, A., Kairys, V., Parkkila, S., Matulis, D., Fluorinated benzenesulfonamide anticancer inhibitors of carbonic anhydrase IX exhibit lower toxic effects on zebrafish embryonic development than ethoxzolamide. *Drug and Chemical Toxicology.* **40** (3), 309-319 (2017).
26. Howe, K. et al. The zebrafish reference genome sequence and its relationship to the human genome. *Nature.* **496** (7446), 498-503 (2013).
27. Granato, M., Nusslein-Volhard, C. Fishing for genes controlling development. *Current Opinion in Genetics & Development.* **6** (4), 461-468 (1996).
28. Bambino, K., Chu, J. Zebrafish in Toxicology and Environmental Health. *Current Topics in Developmental Biology.* **124**, 331-367 (2017).
29. Goldsmith, P. Zebrafish as a pharmacological tool: the how, why and when. *Current Opinion in Pharmacology.* **4** (5), 504-512 (2004).

Figure 1

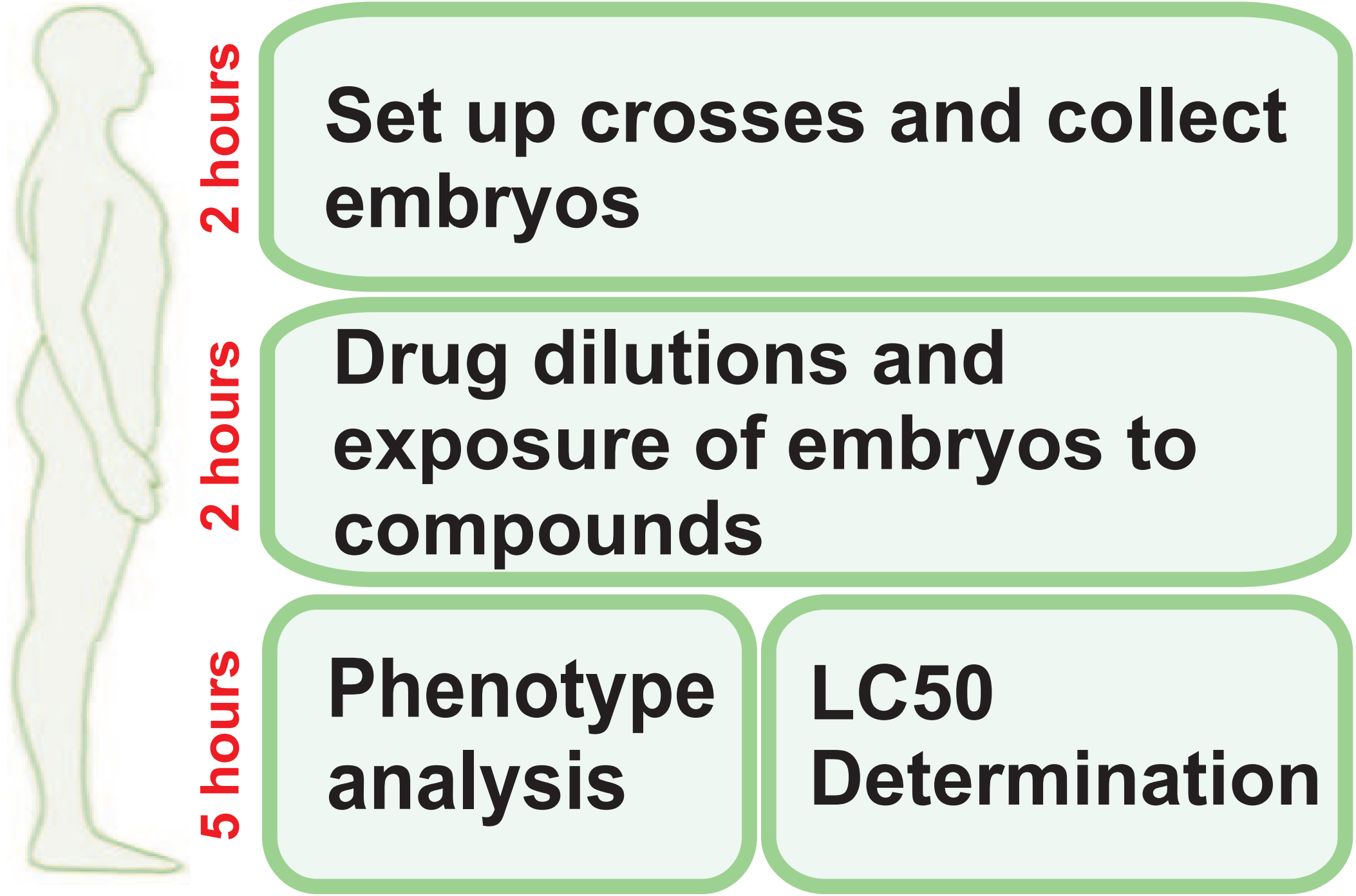


Figure 2

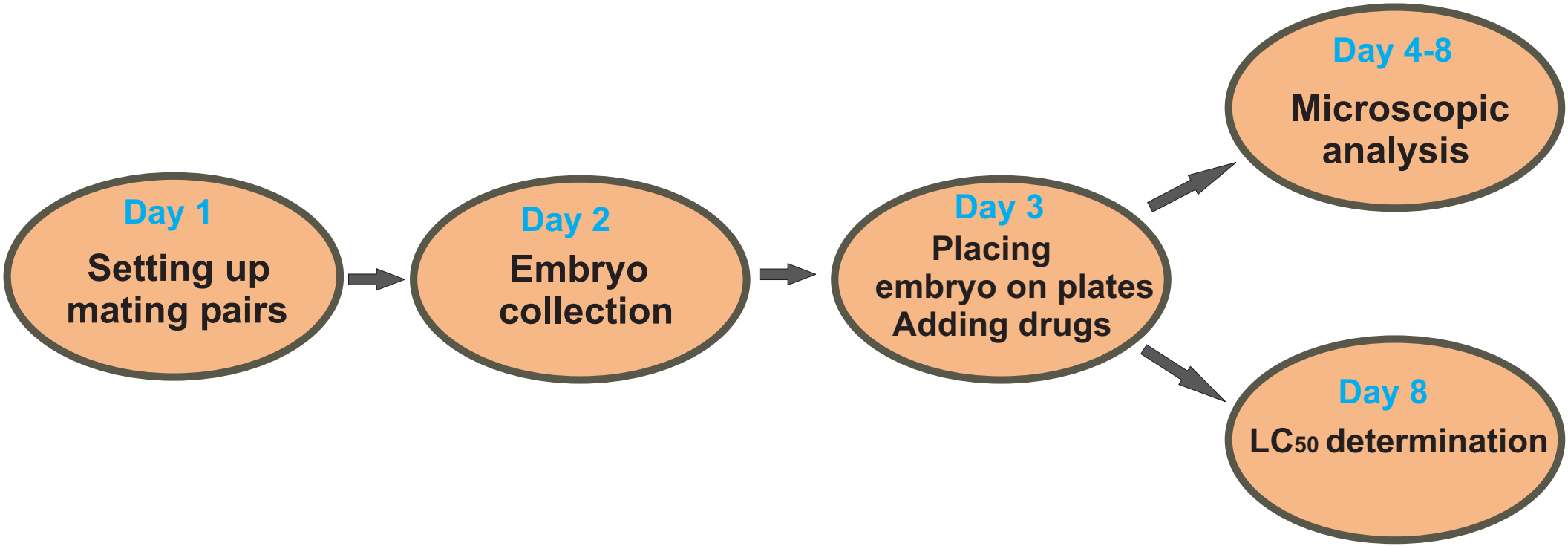


Figure 3

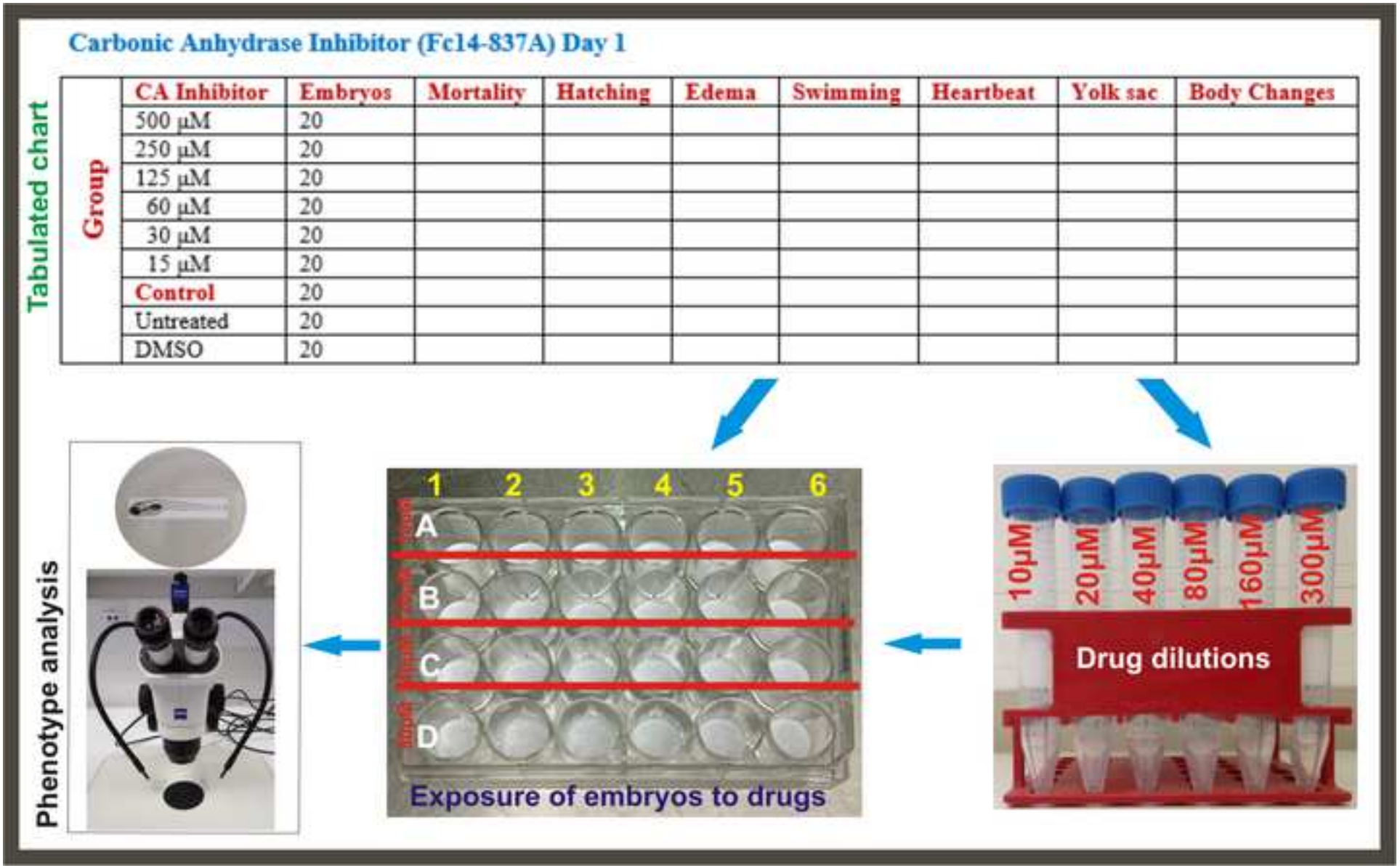


Figure 4

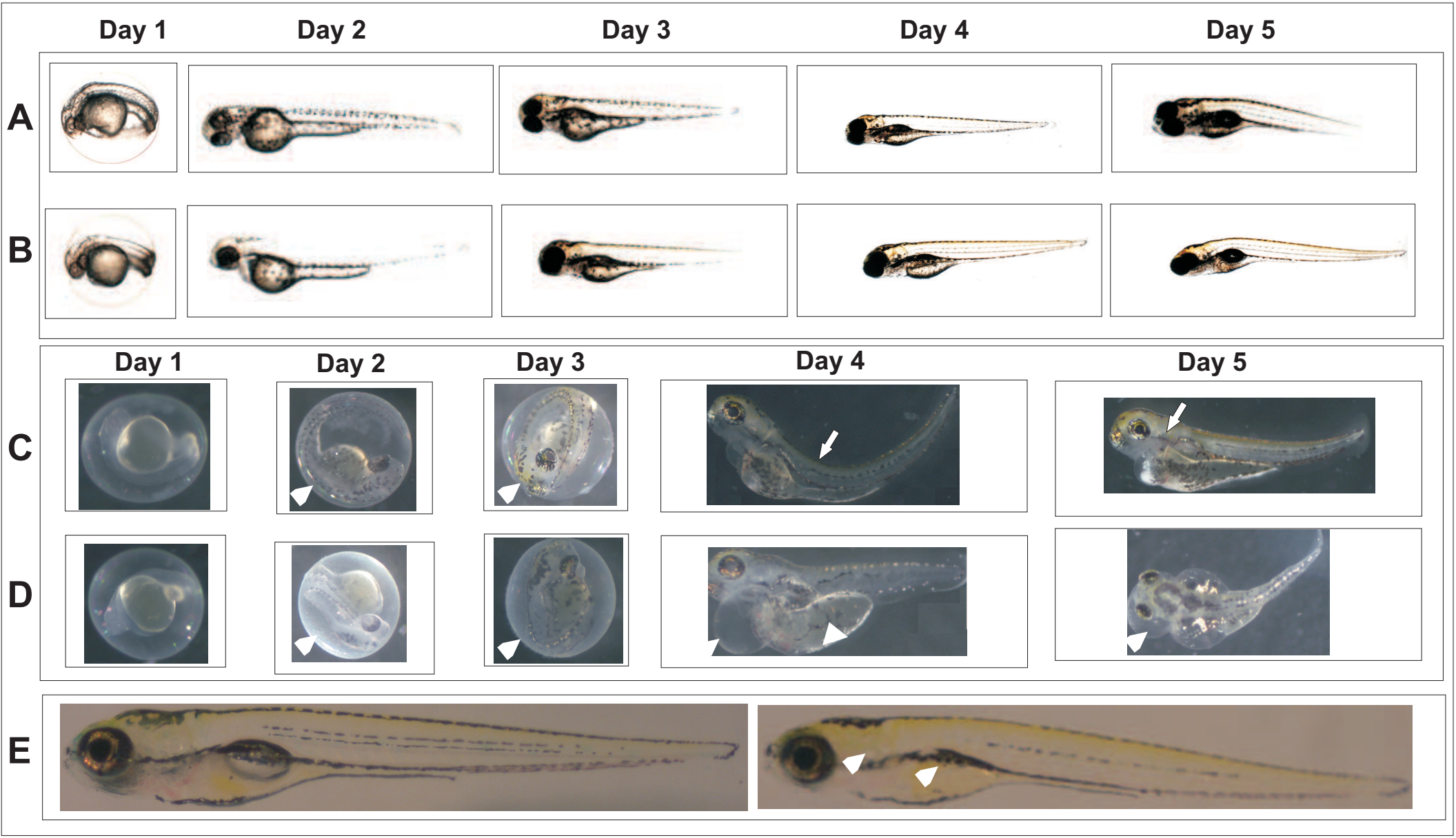


Figure 5

Click here to access/download;Figure;Figure 5_modified_EPS.EPS

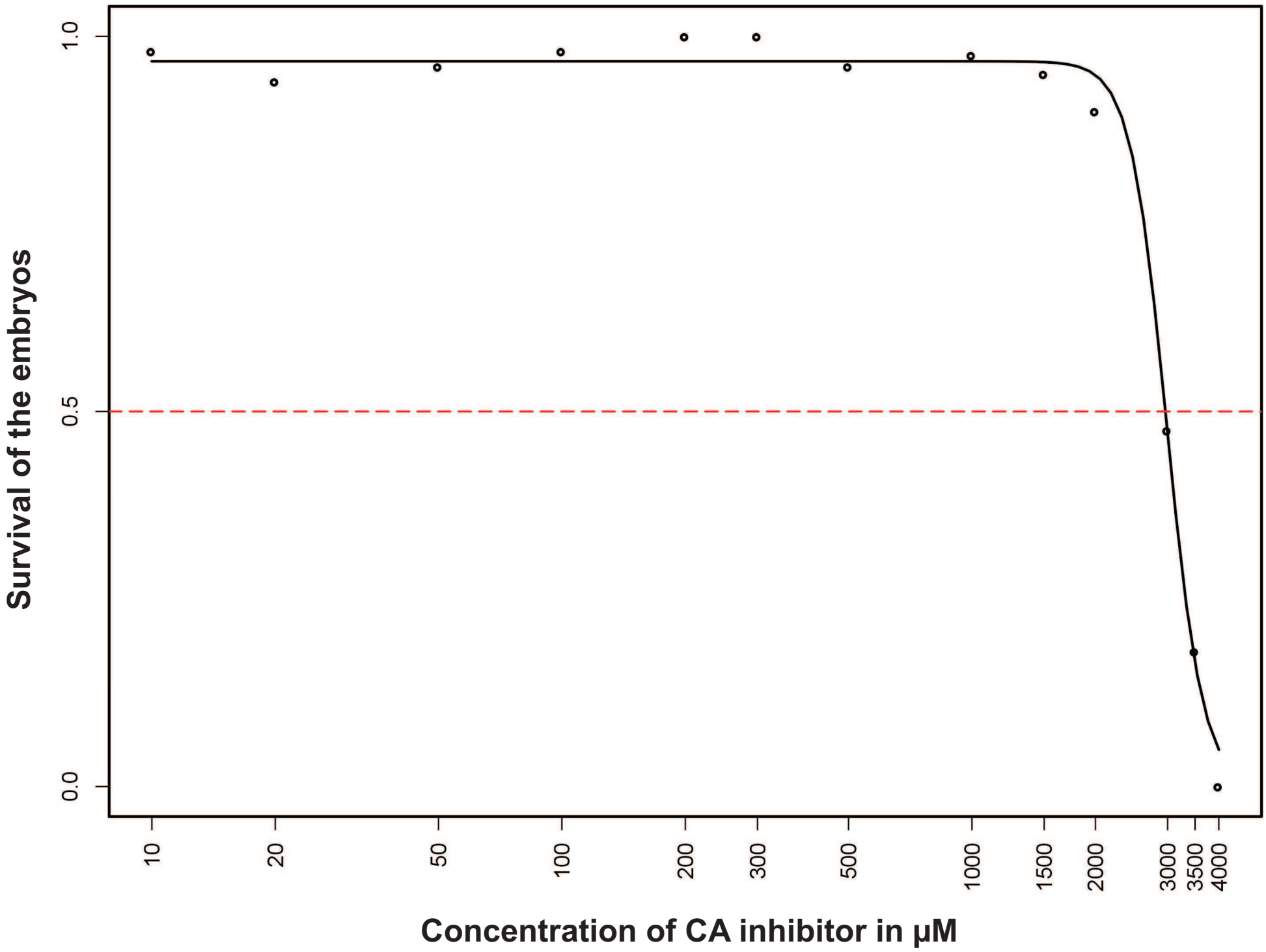
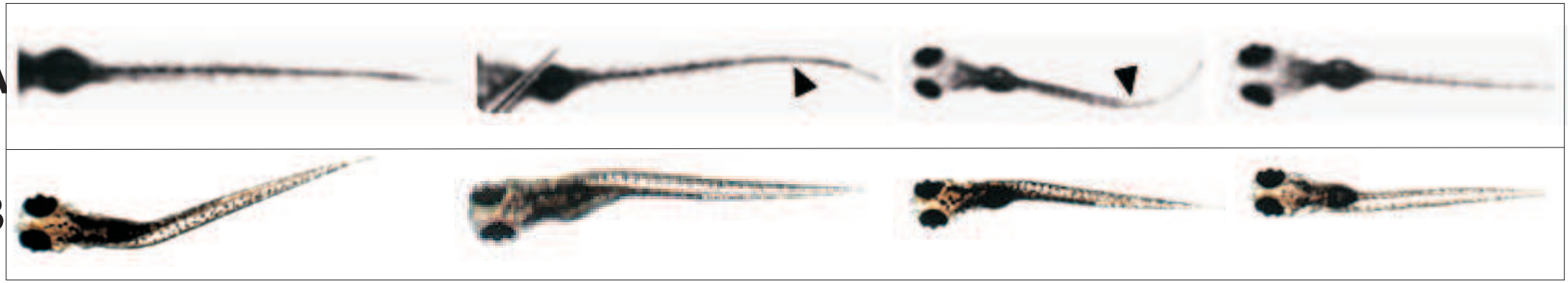


Figure 6



CA inhibitor	**Toxicity Screening**	***LC*** $_{50}$	***In vivo***	**Toxic CAIs**
Carbamates	24	All	1[a]	3[b]
Coumarins	10	All	-	2
Nitroimidazoles	2	All	2	2[c]
Sulfonamides	5	All	-	3
Total	41	All	3	10

aBased on the toxicity screening, the inhibitor was used for inhibition of Mycobacterium in zebrafish model. bLethal inhibitor, cNeurotoxic above 300 µM concentration

Reference
[24], Unpublished
Unpublished
16
25
-

Name of Material/ Equipment	Company	Catalog Number
24-well plates	Nunc	Thermo Scientific
Agarose	BIO-RAD	161-3102
Balance (Weighing scale)	KERN	PLJ3000-2CM
Balance (Weighing scale)	Mettler Toledo	AB104-S/PH
CaCl2	JT.Baker	RS421910024
Disecting Probe	Thermo Scientific	
DMSO	Sigma Aldrich, Germany	D4540
DMSO	Sigma Aldrich, Germany	D4540
Falcon tubes 15 mL	Greiner bio-one	188271
Hematoxylin	Histolab	1825.00
High molecular weight methylcellulose	Sigma Aldrich, Germany	M0262
Incubator for zebrafish larvae	Termaks	B8000
KCL	Merck	1.04936.0500
Methyl Blue	Sigma Aldrich, Germany	28983-56-4
Methyl Blue	Sigma Aldrich, Germany	28983-56-4
MgSO4	Sigma Aldrich, Germany	M7506
Microcentrifuge tubes	Starlab	S1615-5500
Microscope slides SuperFrost Plus	Thermo Scientific	J1800AMNZ
NaCl	VWR Chemicals	27810.295
Paraffin Histoplast IM	Thermo Scientific	8331
Pasteur pipette	Sarstedt	86.1171
Petri dish	Thermo Scientific	
Petri plates	Sarstedt	82.1473
Pipette (1 mL and 200 µL)		
Plates 24-Well	Thermo Scientific	142485
Steriomicroscope/Camera	Zeiss	Stemi 2000-C/Axiocam 105 color
Vials (1.5 mL)	Fisherbrand	
Zebrafish AB strains	Fisherbrand	

ents/Description

jove JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A Method for Evaluation of Drug Toxicity using Zebrafish Embryos

Author(s): Ashok Aspatwar, Milka Hammarèn, Mataleena Parikka and Seppo Parkkila

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ 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-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/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. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

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. 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.

9. 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.

10. 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 discretion and without 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.

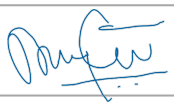
11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and 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 contamination due 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.

12. Fees. To cover the cost incurred for publication, JoVE 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.

13. 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 required per submission.

CORRESPONDING AUTHOR:

Name:	Ashok Aspatwar
Department:	Faculty of Medicine and Health Technology
Institution:	Tampere University
Article Title:	A Method for Evaluation of Drug Toxicity using Zebrafish Embryos
Signature:	
Date:	29.10.2018

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

1) Upload a scanned copy of the document as a pfd 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 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

To
The Editor,
JoVE

6.4.2019

Dear Sir/Madam,

Thank you very much for the invaluable comments from the editorial office of the JoVE Below are the point by point answer to the comments made by editorial office.

The affiliations
Answer: The affiliations are corrected.

The reason for keeping the embryos at 28.5°C?
Answer: Zebrafish embryos are always maintained at 28.5 °C under laboratory conditions.

Do you age match the embryo before plating? If yes, how?
Answer: We age match the embryos. The embryos are collected in the morning and per batch (for three adult breeding pairs) about 300 embryos are obtained which are of same age.

How the time point of 24 h is obtained for examination every 24 h for the experiment
Answer: The time point of 24 hours is calculated from the time of exposure to the chemical. e.g. If the embryos are exposed at 10 am on a given day, the next day 10 am is 24 hours after exposure.

Citation for assessing the parameters.
Answer: The citation is added. Validation in Support of Internationally Harmonized OECD Test Guidelines for Assessing the Safety of Chemicals. Adv Exp Med Biol. 2016; 856:9-32.

Petri dish containing 3% high molecular weight methyl cellulose using a metal probe.
Answer: The 3% methyl cellulose (high molecular with) is a viscous liquid needed for imbedding the fish with a required orientation for microscopic examination. For orienting the fish in this liquid, a metal probe is needed. In the zebrafish laboratory metal probe is routinely used and methyl cellulose is needed for orienting the embryos as per the need for examination and imaging.

Enter all the observations in a table each day either in an online table or on a printed sheet.
Answer: The entries are made manually either on word file with a table or a tabulated paper sheet. This step does not require expensive software.

Do you perform any software analysis for the same or this is done manually? If using software, please provide all the button clicks, graphical user interface, codes etc.
Answer: We assess the toxicity of a chemical and hence we compare the swim pattern of the larvae treated with a particular concentration (which does not induce phenotypic changes) and compared to control group larvae. We check the quality of swimming under microscope and take a video if needed. Does not require the software as it is large scale preliminary toxicity assessment.

Calculation of the LC50 concentration of a chemical compound
Answer: For one concentration of the chemical we use 20 embryos. We calculate the LC50 value

of the concentration at which 50% of the embryos die at the end of the experiment, i.e. after 5 days of exposure to the chemical. e.g. If we are testing 5 concentrations (10μM, 20 μM, 40 μM, 80 μM, 160 μM) using 24 embryos in a 24 well plate (Note: minimum embryos required for one concentration is 20) and at the end of 5 days of exposure 12 embryos are dead at the concentration of 80 μM, then LC50 of the compound in question is 80 μM.

Please explain the defects observed in each case.
Answer: The relevant information is added in the manuscript.

Dilutions required for each compound.
Answer: Changes are made in the main text.

For all the images with microscope, please include a scale bar.
Answer: The images were taken from different studies conducted at different time periods (during the last four years of toxicity screening) using the different microscopes. The rapid toxicity screening of chemical compounds in our study is focused on quick identification of the compounds that are harmful to the developing embryos using simple equipment. The images were taking using simple microscopes with 25 x and 40x lenses and stereomicroscopes by zooming in and zooming out to identify phenotypic changes in most of the studies and in couple of compounds we identified defects in swim pattern. In such cases, it is difficult to keep record of scale and also not needed. More importantly, to get the scales will be arbitrary and hence difficult to provide the correct scales for the past studies.

Thanking you very much.

With Best Regards,

Ashok

Journal

Journal of Enzyme Inhibition and Medicinal Chemistry ›

Volume 33, 2018 - Issue 1

Research Paper

Nitroimidazole-based inhibitors DTP338 and DTP348 are safe for zebrafish embryos and efficiently inhibit the activity of human CA IX in *Xenopus* oocytes

Ashok Aspatwar, Holger M. Becker, Nanda Kumar Parvathaneni, Milka Hammaren, Aleksandra Svorjova, Harlan Barker, ...show all

Pages 1064-1073 | Received 30 Apr 2018, Accepted 25 May 2018, Published online: 17 Jun 2018

Download citation https://doi.org/10.1080/14756366.2018.1482285 Check for updates