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Set-up of an infrared fast behavioral assay using zebrafish (*Danio rerio*) larvae, and its application in compound biotoxicity screening

Darío Bichara[a,b], Nora B. Calcaterra[b], Silvia Arranz[b], Pablo Armas[b]* and Sergio H. Simonetta[a]*

ABSTRACT: Zebrafish (*Danio rerio*) is increasingly employed for evaluating toxicity and drug discovery assays. Commonly experimental approaches for biotoxicity assessment are based on visual inspection or video recording. However, these techniques are limited for large-scale assays, as they demand either a time-consuming detailed inspection of the animals or intensive computing resources in order to analyze a considerable amount of screenshots. Recently, we have developed a simple methodology for tracking the locomotor activity of small animals cultured in microtiter plates. In this work, we implemented this automatic methodology, based on infrared (IR) microbeam scattering, for measuring behavioral activity in zebrafish larvae. We determined the appropriate culture conditions, number of animals and stage of development to get robust results. Furthermore, we validated this methodology as a rapid test for evaluating toxicity. By measuring the effects of reference compounds on larvae activity, we were able to estimate the concentration that could cause a 50% decrease in activity events values (AEC_{50}), showing a strong linear correlation ($R^2 = 0.91$) with the LC_{50} values obtained with the standard *Dar*T test. The toxicity order of the measured compounds was $CuSO_4$ > 2,4-dinitrophenol > 3,4-dichloroaniline > SDS > sodium benzoate EDTA > K_2CrO_4; regarding solvents, EtOH ≈ DMSO. In this study, we demonstrate that global swimming behavior could be a simple readout for toxicity, easy to scale-up in automated experiments. This approach is potentially applicable for fast ecotoxicity assays and whole-organism high-throughput compound screening, reducing the time and money required to evaluate unknown samples and to identify leading pharmaceutical compounds. Copyright © 2013 John Wiley & Sons, Ltd.

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Keywords: zebrafish; *Danio rerio*; biotoxicity; tracking method; high throughput screening; automated assay; behavior

Introduction

The production of chemical compounds has increased worldwide from 1 million tons to more than 400 million tons per year in the pasts 80 years (Vogelgesang, 2002). The production, use and final disposal of these anthropogenic compounds lead, in different extents, to their environmental presence contributing to pollution. In particular, the entry of contaminants to aquatic habitats could be associated with the discharge of sewage networks, industrial effluents, accidental spills or agriculture activity; and it can alter, temporarily or permanently, the ecological balance. Numerous methods have been standardized for water pollutant measurement (Gilcreas, 1966). Although most of them employ physical and chemical readouts, the presence of unknown toxic molecules in samples can be detected by measuring adverse physiological effects on freshwater organisms (Scott and Sloman, 2004).

One of the organisms currently used in toxicity assays is the zebrafish *Danio rerio*, a small cyprinid naturally found in the Ganges River in South-East Asia (Eaton and Farley, 1974). The zebrafish lives in tropical freshwater and measures 3 to 5 cm as an adult, and 1 cm at the larval stage. Some characteristics of this species, such as a high breeding rate (a single mature female lays 50–200 eggs per day) (Laale, 1977), easy view of embryogenesis and organ development and low cost, have defined it as a major laboratory model in development and general molecular biology. In addition, because of other properties including small size, fast and external development of the embryos, genomics databases, available molecular tools and a relative high human-gene ontology, it is becoming an excellent model for human diseases and a rewarding tool for drug screening (Ackermann and Paw, 2003; Briggs, 2002; Grunwald and Eisen, 2002; Liu and Leach, 2011; Scholz *et al*., 2008).

Fish have traditionally been considered an indispensable component of toxicity testing strategies. The fish acute toxicity test (OECD, 1992) was of major importance in the past decades. However, considerations of animal welfare have increasingly questioned ecotoxicity testing using adult fish and stimulated the development of alternative assays. In view of this, the embryo test with *Danio rerio* (*Dar*T) was set-up (Nagel, 2002), replacing the traditional fish acute toxicity test (Braunbeck and Lammer,

*Correspondence to: Sergio H. Simonetta, Patricias Argentinas 435, (C1405BWE), Buenos Aires, Argentina. E-mail: ssimonetta@leloir.org.ar; Pablo Armas, Suipacha 590, (S2002LRL), Rosario, Argentina. E-mail: armas@ibr.gov.ar

[a]Fundación Instituto Leloir, CONICET, Buenos Aires, Argentina

[b]Instituto de Biología Molecular y Celular de Rosario, CONICET; and Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina

2005, 2006; ISO 15088:2007, 2007; OECD, 2006). *Dar*T is based on inspecting lethal and sub-lethal developmental endpoints of zebrafish embryos cultured in 24-well microplates. However, it requires a considerable amount of handwork, trained examination of the embryos and it is not practically applicable when a high number of samples have to be measured in a short time. Some modifications were introduced to improve the speed of toxicity measurements; for example, the use of a 96-well microplates or high throughput video recording (Braunbeck and Lammer, 2006; Parng *et al*., 2002). Even so, time-intensive handwork or large computational resources are still required.

Screening and high throughput studies on compounds toxicity usually involve high-cost processing systems capable of analysis of large data series, so that the development of new technologies in the area might be useful in overcoming these limitations. Recently, we designed a system based on infrared (IR) light detection capable of easily tracking locomotor activity and the circadian behavior of *Caenorhabditis elegans* cultured in 96 and 384 wells (Simonetta and Golombek, 2007) and we realized that, with minor adjustments, it was potentially applicable to quantify the global activity of small aquatic vertebrates.

In this study, we demonstrate that it is possible to quantify zebrafish larvae swimming activity with an IR array sensing device, allowing compounds toxicity in vertebrates to be studied in a simple and fast manner, without requiring trained personal or manual scoring, and possibly be applied to high throughput analyzes of different kind of samples.

Material and Methods

Animal Maintenance and egg Production

A breeding stock of heterogeneous wild-type (WT) zebrafish was purchased from a local pet shop and inbred in our facility as described by Kimmel *et al*. (1995). Sexually-mature females and males (8–12 months old) were kept at a ratio of 3:1 in a glass aquarium filled with filtered tap water at 26 ± 1 °C under a 14-h day/night light regime [light ON = eitgeber Time zero (ZT0)]. Fishes were fed with dry flakes (TetraMin PRO®) twice per day and with nauplia larvae of *Artemia spec*. once a day *ad libitum*. For embryo production, four females and two males were crossed the night before the spawning day in traps made of plastic mesh, to prevent the eggs from cannibalism. Green algae and glass marbles were also added as a spawning substrate. Collected eggs were maintained in a Petri dish with E3 saline embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$ and 0.33 mM Mg_2SO_4) at 28.5 °C, and examined for fertilization under a Nikkon TN-PSE80 stereomicroscope. Non-fertilized eggs were discarded.

Experimental Design

All animals were handled in compliance with relevant national and international guidelines, and experiments were designed without interfering with current animal welfare legislation.

Embryo Test with Zebrafish

The *Dar*T toxicity test was carried out according to previously described protocols (OECD, 2006; Nagel, 2002; Schulte and Nagel, 1994). In brief, fertilized embryos were transferred to a 24-well plate filled with 2 ml of the tested solution per well (1 embryo in each well) and subjected to five different concentrations (20 technical replicates of each concentration plus distilled water as control). Embryos and larvae were visualized under a dissecting microscope at 8, 24 and 48 h post fertilization (hpf). Lethal (coagulation, tail not detached, no somites and no heart beat) and sub-lethal (completion of gastrula, formation of somites, development of eyes, spontaneous movement, heart-beat/blood circulation, pigmentation and edema) endpoints were recorded. The concentration required to kill half the members of a tested population after specified test duration (LC_{50} value) was calculated using the DEBTox software, which uses the Probit method for analysis (http://www.bio.vu.nl/thb/deb/deblab/debtox/). Seven Q4 reference drugs and two commonly used solvents were tested. Three different sets of animals were used to perform three independent experiments.

Reference Drugs and Solvents

Copper(II) sulfate (CuSO4), potassium chromate (K_2CrO_4), 2,4-dinitrophenol, 3,4-dichloroaniline, sodium benzoate and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, catalog no. C1297, 216615, D19850-1, 437778, B3420 and D8418, respectively; and sodium dodecyl sulfate (SDS) was obtained from Bio-Rad catalog no. 161-0302; ethylenediaminetetraacetic acid (EDTA) and ethanol (EtOH) were purchased from Anedra, catalog no. 6057 and 9516, respectively.

Modified *Dar*T Assay

The modified *Dar*T assay was carried out in 96-well multiplates instead of 24-well plates, using a final volume of 200 µl per well. Five serial dilutions of the tested compound (16 wells per each dilution) were used plus distilled water as a control. Results were recorded as described above.

Automated Measurement of Larvae Activity Events

The system is based on an IR microbeam arrangement that detects light refraction through the zebrafish body, essentially as described elsewhere (Simonetta and Golombek, 2007). Animals were placed in 96-well microplates and subjected to illumination with two IR microbeams per well (100 µm wide and of 880 nm wavelength each). A transient fluctuation in the signal is generated when larvae move across the light beam and received by a phototransistor array. Light signals output were digitalized by a multichannel ADC system (WMicrotracker, Designplus SRL, Argentina) at a sample rate of 10 samples per Q6 sec and a 10-bit resolution. Data were acquired with an IBM-PC connected via a RS232 protocol and processed by dedicated software programmed in MS-Visual Basic. Signal activity events (defined as the times that larvae cross through IR microbeams) were calculated in real time by detecting small fluctuations in the received signal. Variations greater than 3% in the received signal (empirically determined threshold capable of detecting larvae IR microbeam interruption, but not basal electronic noise) were considered as activity events. Fish swimming activity was calculated summing up the number of activity events during a period of 15 min. Data were reported as the averaged activity event recorded for each microbeam pair ± standard error of the mean (SEM).

Automated Measurement of Compounds Toxicity

Three 48-hpf non-hatched zebrafish embryos were placed in each well of a 96-well plate containing 225 µl of E3 medium and incubated for additional 48 h at 28 °C. Twenty-five mircoliters of a 10-fold concentrated compound solution or E3 medium (control) were added to complete a 250-µl final volume. For each assay, eight technical replicates were used for each dilution. Activity events were recorded during 15 min at 4, 24 and 48 h after the addition of the tested compound at room temperature and immediately after, the plates were checked by manual inspection at a stereoscopic microscope, in order to validate the activity count. Experiments were initiated between 3 and 5 h after ambient illumination went on (ZT3 to ZT5). The concentration of the compound that causes a 50% decrease in activity event values (arbitrarily defined as AEC_{50}) was calculated by plotting a relative activity event vs. compound concentration. Curves were fitted to 3-parameter sigmoid or logistic equations. Graphics and statistical comparison based on a linear regression model, 95% confidence and prediction interval bands were calculated using SigmaPlot®. The no observed effect concentration (NOEC) was calculated with ANOVA and Dunnet's test ($P = 0.05$).

Results

Automated Measurement of Zebrafish Larvae Movement Activity

Movement activity events were detected by subjecting 4 dpf zebrafish larvae to IR microbeams (similar to as described in Simonetta and Golombek, 2007). As is shown in Fig. 1A, transient changes in the recorded signals were observed as the animals moved through the beams. A linear correlation in behaviour quantification values was obtained as the number of larvae per well increased from 1 to 4 ($R^2 = 0.996$; Fig. 1B). Although fish larvae tend to adopt a quiescent state in constant darkness (Burgess and Granato, 2007), measurement of the first 15 min has shown a good reproducibility and consistent activity [$Activity_{(t0–15min)} = 77.2 \pm 2.3$; $Activity_{(t15-30min)} = 40.1 \pm 2.1$; $n = 200$ measured at 4 dpf, $P < 0.001$ *t*-test).

In order to discard possible interference with changes in behavioral activity associated with circadian rhythms (Padilla *et al.*, 2011), swimming behavior was recorded between 3 and 8 h after lights went on, and control animals were included in each test. No statistical differences were found in control animals at different testing times [$Activity/15\ min_{(ZT3)} = 83.7 \pm 3.4$; $Activity/15\ min_{(ZT8)} = 74.9 \pm 3.3$; $n = 85$, $P > 0.05$ *t*-test].

Activity Events as Readout for Toxicity Measurement

The next challenge was to assess the potentiality of the adapted system to perform automated toxicity assays. Those were carried out by measuring the toxic effects of seven reference compounds and two solvents previously assayed by *Dar*T (Braunbeck *et al.*, 2005). Moreover, a *Dar*T in a 96-well microplate was performed in parallel as a reference (Table 1). T1

We found a consistent reduction in activity events as the tested drugs concentration increased (Fig. 2). Most representative results were obtained 48 h post-exposure (hpe), similar to previously described for *Dar*T assays (Braunbeck and Lammer, 2005; Nagel, 2002; Schulte and Nagel, 1994). However, a short-time exposure, such as 4 hpe, was enough to produce drastic effects on motility in at least 50% of tested compounds at a low concentration (see Fig. 2: DNP, DCA, K_2CRO_4 and DMSO treatment). The NOEC value of EtOH and DMSO was 1% v/v for both solvents. Noteworthy, a high degree of correlation [$R^2 = 0.91$, 78% of values within the confidence belt ($P < 0.05$) and 100% of them inside the prediction range] was observed between AEC_{50} values measured at 48 hpe and LC_{50} values obtained using *Dar*T (Table 1 and Fig. 3). In addition, the whole test extent decreased from 75 min in *Dar*T (15 min for placing embryos + 15 min for compound pipetting + 45 min for recording endpoints per compound) to just 30 min using the tracking system (the time necessary for placing embryos and dosed compounds in 96-well microplates). Furthermore, using the IR screening system we were capable of measuring different compounds at the same time and even reading 384-microwell plates (Supplementary Fig. 1). F2 F3

Discussion

Zebrafish is becoming popular as a biosensor for ecotoxicity studies and drug toxicity assays. During the past decade, standardized tests have been validated and accepted by ISO

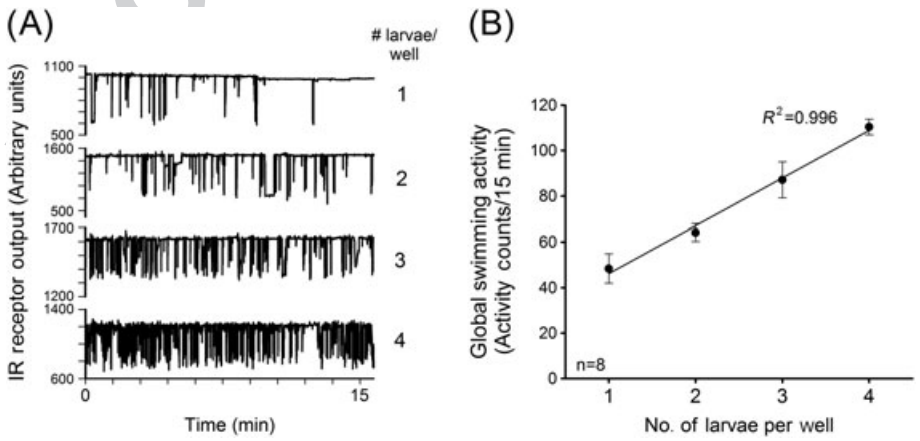

Figure 1. Correlation between measured activity and population size. (A) zebrafish larvae [4 days post-fertilization (dpf)] were cultured in 96-well plates and subjected to infrared microbeam lights (100 um wide, power < 1 mW). Representative plots of the signal obtained from photoreceptors output is shown against time. Activity events, observed as sharp signal fluctuation, are detected as the larvae swim through the microbeams. (B) A linear correlation is appreciated between activity quantification, defined as the accumulation of events counting per 15 min, and the number of larvae per well. Plot corresponds to average of eight experimental replicates ± standard error of the mean (SEM).

Table 1. LC_{50} values of reference compounds for the standard *Dar*T and EAC_{50} values for automated swimming activity measurement.

Table 1. Obtained toxicity values of reference compounds [mg/L]

	LC_{50} *Dar*T test		
Compound	Published value[a]	Data from our laboratory	AEC_{50} MicroTracker
$CuSo_4$	0.49	0.27 ± 0.03	1.64 ± 0.16
2,4-dinitrophenol	0.90	0.60 ± 0.06 $(0.61 \pm 0.01)^{(3)}$	4.40 ± 0.35
3,4-dichloroaniline	2.40	3.76 ± 0.66	8.76 ± 0.01
SDS	8.81	16.1 ± 0.97	36.30 ± 1.90
Sodium Benzoate	36.60[b]	461 ± 123	68.50 ± 11.50
EDTA	727	1883 ± 309 $(1395 \pm 145)^{c}$	260.33 ± 12.39
K_2CrO_4	-	4680 ± 381	1995.00 ± 544.16
Ethanol	1.41 % v/v	1.21 ± 0.20 % v/v	1.87 ± 0.70 % v/v
DMSO	2.65% v/v	1.88 ± 0.72 % v/v	1.59 ± 0.01 % v/v

[a]Data reported by Braunbeck and Lammer (2005) and Nagel (2002).
[b]Value for benzoic acid.
[c]Data for modified *DarT* in 96-well plates are expressed between brackets. Average of three independent set of experiments ± standard error of the mean (SEM).

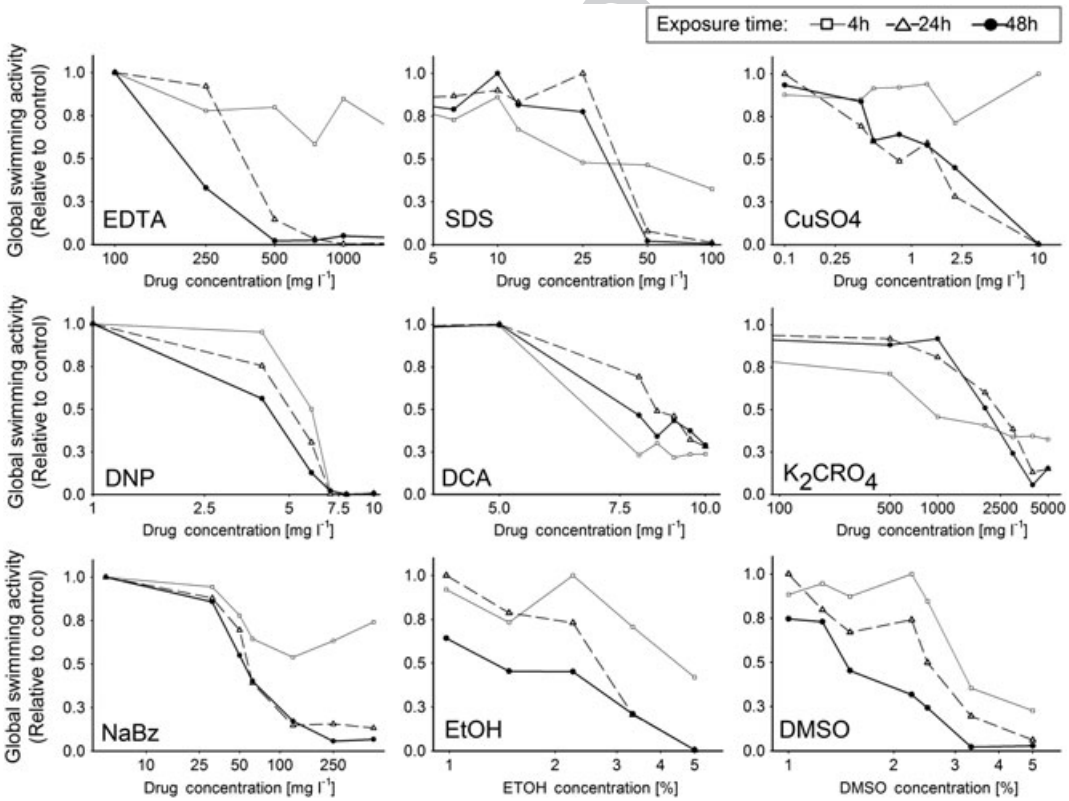

Figure 2. Dose-response curves for nine reference toxic treatments measured at different times post-exposure. Swimming activity of zebrafish larvae (normalized events per 15 min) decreases as the compound concentration increases. AEC_{50} is determined as the concentration of compound necessary to reduce 50% the swimming behavior. Chemical abbreviations: EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; $CuSO_4$, copper (II) sulfate; DNP, 2,4-dinitrophenol; DCA, 3,4-dichloroaniline; K_2CrO_4, potassium chromate; NaBz, sodium benzoate; EtOH, ethanol; DMSO, dimethyl sulfoxide. The drug axis is plotted in a logarithmic scale. Average ± standard error of the mean (SEM) is shown ($n = 8$).

standards and official organisms (OECD and TOXCAST). Although toxicity tests based on manual/eye inspection screenings have been preferentially standardized, the potentiality of this animal model for fast and automated toxicity screenings has not been totally exploited yet. The adaptation of *Dar*T to 96-well microplates (Braunbeck *et al.*, 2005) or even a smaller format such as microfluidics cameras (Lammer *et al.*, 2009) were successfully employed. However, time-consuming trained-eye endpoints inspection is still necessary.

In the past years, several methods were developed to improve the application of fast experiments/miniaturization for high-speed semi-automatic detection of zebrafish behavior and

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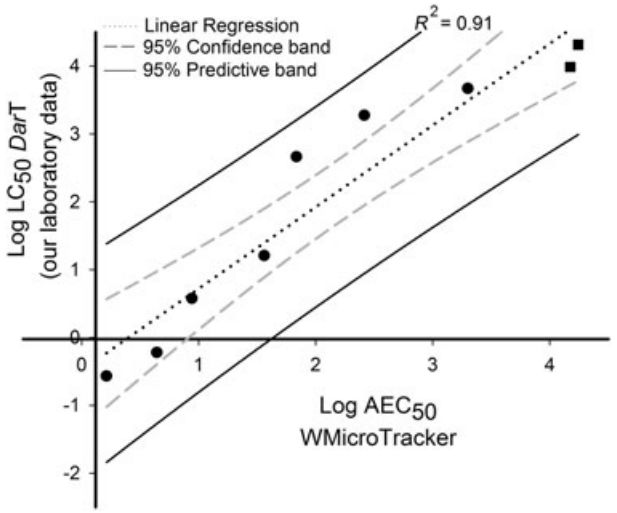

Figure 3. Linear correlation between the *Danio rerio* embryo toxicity test (*DarT*) and the automated swimming recording assay (WMicrotracker) for nine reference toxics. Compounds (circles) and solvents (squares) are expressed in (mg L^{-1}). Data of three independent sets of experiments per compound were grouped and plotted logarithmically. For statistical purpose, a 95% confidence interval and prediction interval are shown.

chemical compounds effects (Orger *et al*., 2004; Zon and Peterson, 2005). Automatic video detection is preferable to any other methodology; however, technical and technological limitations in fully automated video recognition, real-time analysis and plate scanning are still bottlenecks in the automation of such a process (Letamendia *et al*., 2012).

Here we report the successful adaptation of a tracking system previously described for recording *C. elegans* circadian rhythms to be used for automatically quantifying zebrafish activity (swimming) events. Our approach is based on the probability of larvae crossing through microbeam rays, thus the output is not a direct measurement of activity. This issue could be appreciated as a main limitation. However, the light beam interruption rate has been shown to correlate with normal healthy behavior in different animal models, and this approach has been used for years to study several biological issues, such as *Drosophila* circadian rhythms and neurodegenerative disorders, adult fish and rodent studies (Nelson *et al*., 2002; Rezaval *et al*., 2008; Rosato and Kyriacou, 2006; Young *et al*., 1993).

In addition, we show that our system is useful for measuring lethal/sub-lethal chemical compounds toxicity. With this procedure, we were able to determine accurately the AEC_{50} of seven drugs and two solvents commonly used as reference compounds in toxicity tests. The degree of toxicity determined by employing our approach strongly correlated with that detected by employing the widely accepted *Dar*T methodology. It is known that the zebrafish behavioral response is not necessarily related to toxic concentrations (Magalhaes *et al*., 2007). However, in spite of this, many previous studies have demonstrated the usefulness of quantifying fish swimming activity (referred as activity events in this work) as a way of assessing pollutants and compounds toxicity (de Esch *et al*., 2012; Padilla *et al*., 2011; Winter *et al*., 2008). In our case, we found a correlation between activity measurements and *Dar*T test values, with 22% of the compounds (2 out of 9) outside the confidence belt, showing higher toxicity than reported. This particularity could be attributed to a decrease in activity as a sub-lethal effect of the toxicant, as reported in Chen *et al*. (2009). Another possibility is that those results are as a result of a differential response of larvae compared with embryos. Nevertheless, further studies should be carried to clarify these differences.

Zebrafish embryos start moving from 24 hpf onwards; however, coordinated movement (swimming) is achieved at 4 days post-fertilization (dpf) (Kimmel *et al*., 1995). Hence, although we measured embryonic spontaneous movement from 24 hpf onwards (data not shown), thus suggesting that our system is potentially applicable to early staged embryos (in accomplishment with European chemicals policy (REACH), we recorded activity events at 4 dpf stage. This allowed us to abolish possible interferences generated by the low frequency and stochasticity of spontaneous movements in embryo (Saint-Amant and Drapeau, 1998). As stated by Padilla *et al*. (2011), many factors beyond toxicant exposure affect larval zebrafish locomotor behavior, and the control of those variables is needed to promote consistent behavioral assessments and reproducible outcomes. In our study, we covered this issue by optimizing the measurement time, age, acquisition lapse and even the number of larvae per well. These adjustments were enough to provide consistent results among experiments and a good correlation with *Dar*T assay. On the other hand, part of the deviations observed in regression curves could be attributed to the existence of certain biological variability (as shown in Supplementary Fig. 2) and/or to heterogeneity in genetic stocks, as parental zebrafish were purchased from pet shop cultures.

The high-screening capacity of our automated assay is comparable to that obtained using more complex devices. For example, the testing capacity is about 4,500 compounds per month per device, performed by a unique technician at an 8-hour commitment per day. This can be expanded using 384-well plates and automatic embryo dispensers, which are compatible with our device, reaching 18,000 compounds per month and even more using more than one device. Finally yet importantly, the whole process can be automated using a plate stacker allowing it to be used for high-throughput drug screening purposes. Finally, we remark that the same methodology is potentially applicable for neurotoxicity tests or drug discovery as there are plenty of human diseases that are being modeled in zebrafish, as described in previous reports (Barros *et al*., 2008; Flinn *et al*., 2008; McGrath and Li, 2008; Sadler *et al*., 2005).

In conclusion, we present a novel, simple and easy to scale up methodology based on IR microbeam interruption, which could be used in toxicity assays, behavioral experiments, activity measurements and early drug discovery taking advantage of zebrafish larvae or other organisms of a similar size.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

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Competing Interests

S.H.S. is one of the inventors of a filed patent associated with the technology discussed in this study. This does not alter the authors' adherence to the journal policies on sharing data and materials.

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Author Query Form

Journal: Journal of Applied Toxicology

Article: jat_2856

Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions.

- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please refer to the proof mark-up symbols guidelines. If manually writing corrections on your proof and returning it by fax, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

Query No.	Query	Remark
Q1	AUTHOR: Please confirm that the sense of the sentence beginning is now correct: The toxicity order of the measured compounds was. . .'	
Q2	AUTHOR: Please confirm that the correspondence details are all correct.	
Q3	AUTHOR: Please confirm that the affiliation details are correct.	
Q4	AUTHOR: Please check this website address and confirm that it is correct. (Please note that it is the responsibility of the author(s) to ensure that all URLs given in this article are correct and useable.)	
Q5	AUTHOR: Please provide address details including the state if in the USA for the following manufacturers: Sigma-Aldrich, Bio-Rad and Anedra.	
Q6	AUTHOR: Please provide the name of the town/city in Argentina.	
Q7	AUTHOR: Please confirm which is the legend for Table 1.	
Q8	AUTHOR: Journal style is to use unstructured abstracts but you supplied a structured abstract. All structuring has been removed – please check.	
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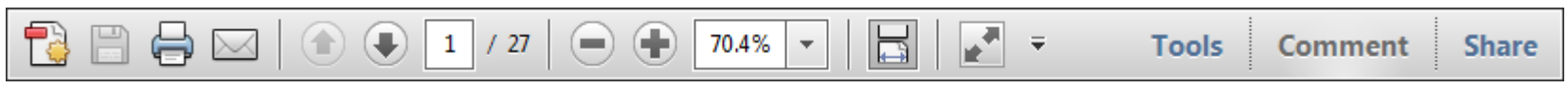
WILEY-BLACKWELL

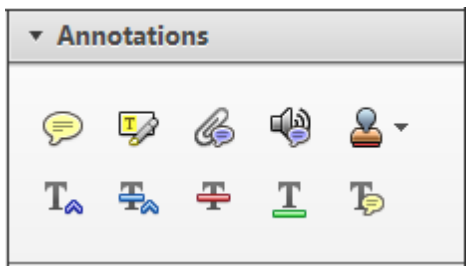
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

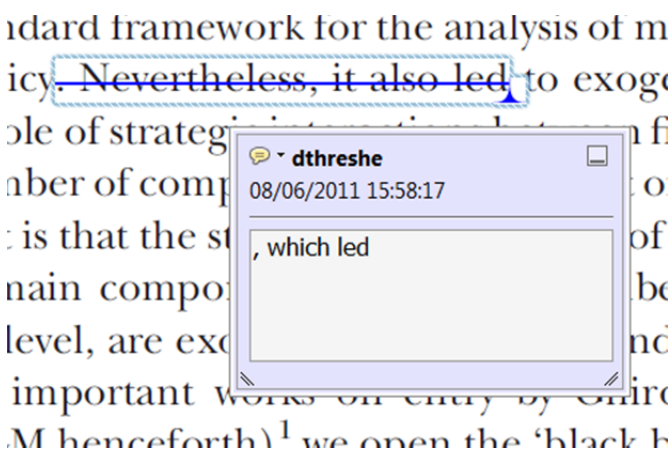


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

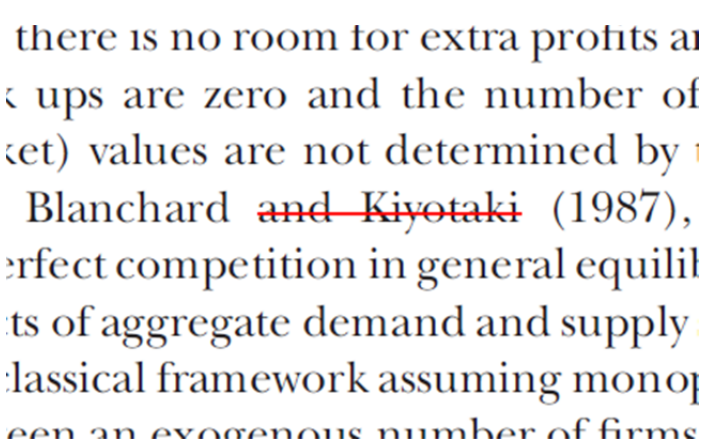


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

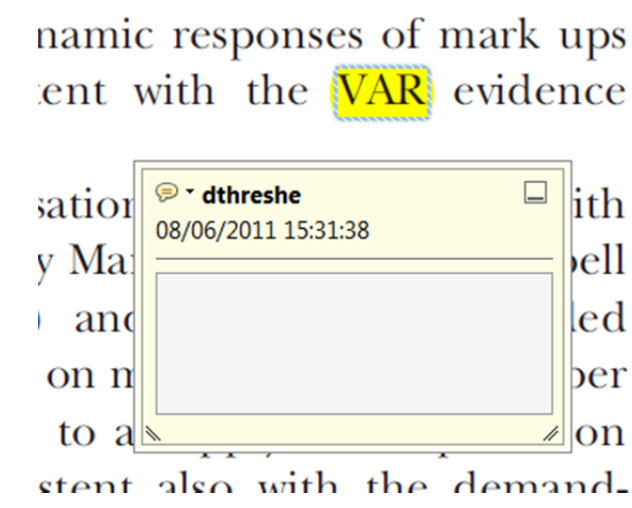


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

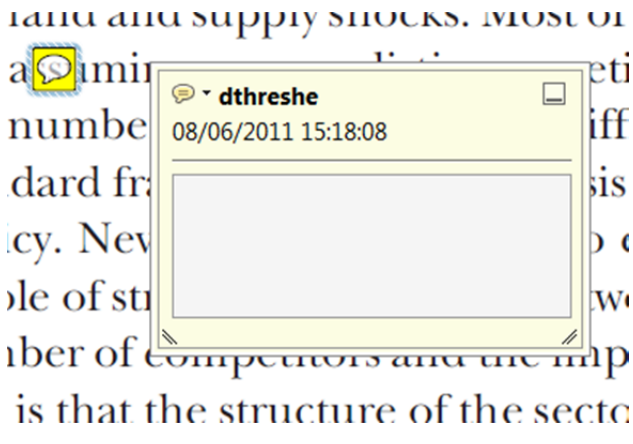


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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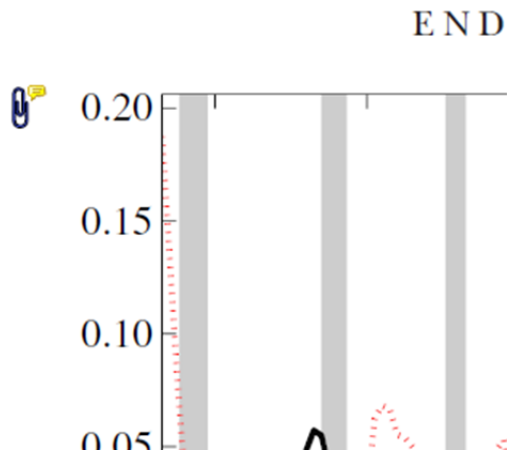
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

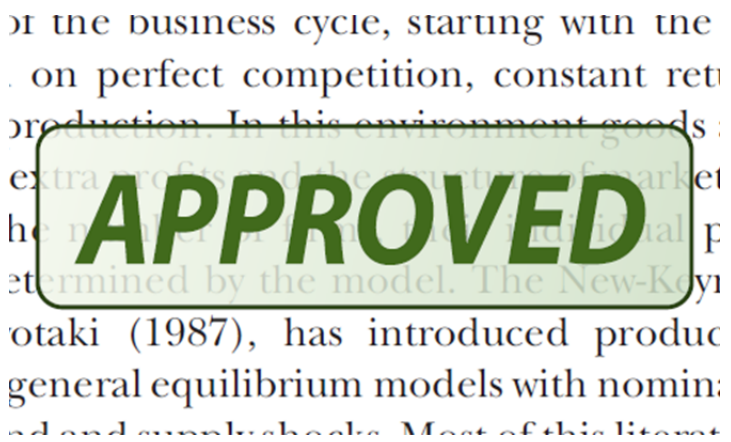


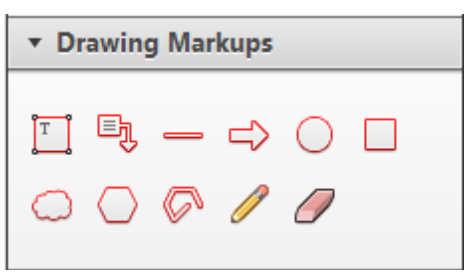
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



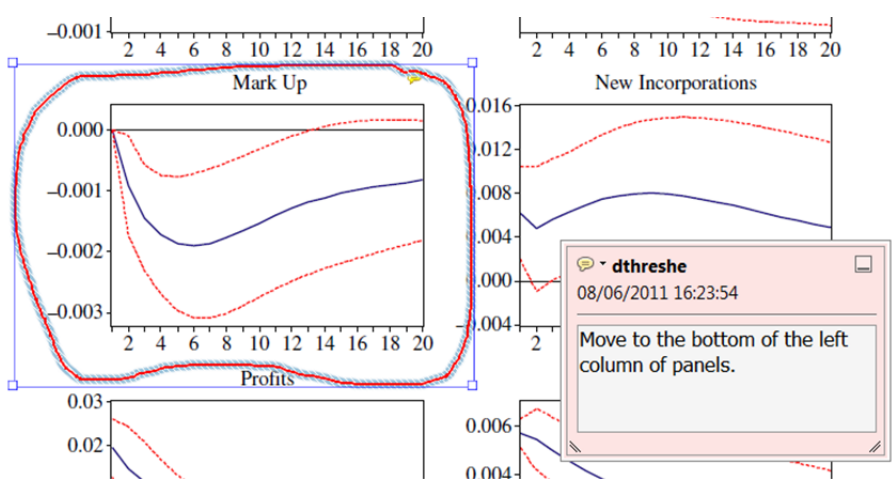


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: