

Multimode Detection

Authors:

Enrico Mingardo, Benjamin Odermatt

Anatomical Institute

University of Bonn

Bonn, Germany

Felix Haeberlein, Jesus Gomeza

Nina-Katharina Schmitt, Evi Kostenis

Institute for Pharmaceutical Biology

University of Bonn

Bonn, Germany

Norbert Garbow

PerkinElmer, Inc.

Hamburg, Germany



High-Throughput Cell Analysis of a Fluorescent Zebrafish Reporter Line Using the PerkinElmer EnSight Multimode Plate Reader

Introduction

High-Throughput Screening (HTS) has been developed for cell-culture-based assays, involving an automatic robotic system to load and analyze 96,

384, 1536 and even 3456 well plates.¹ The HTS consists predominantly of automated testing of large chemical libraries for drug research, analyzing cell phenotype and all the absorbance-based assays. More complex experimental models are used to refine drug discovery and follow the initial *in vitro* investigation. In the last few years, some of the cell-based HTS technologies were adapted to zebrafish (zf) embryos and larvae.² This application note demonstrates how the imaging technology of the PerkinElmer EnSight™ microplate reader can be applied to zebrafish larvae to achieve high-throughput phenotype (HTP) images of 96 larvae (Fig. 1). It also shows how the PerkinElmer Kaleido™ analysis software can detect the number of migrated fluorescent cells under different drug treatment conditions.

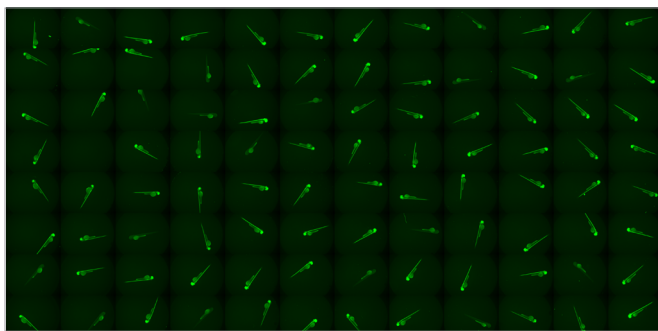


Figure 1. EnSight images of green fluorescence zebrafish larvae in a 96-well plate.

Zebrafish have become one of the most used *in vivo* vertebrate disease models in recent times. Due to its high number of progeny, external fertilization and transparent larvae stadium, zebrafish are a suitable *in vivo* model organism for generating transgenic fluorescent reporter lines used, for example, to screen compounds for drug discovery.

So far, most zebrafish larvae imaging is still performed by conventional fluorescence microscopy, using manual fish-positioning and cell counting.³ This manual method is time-consuming and therefore lacks a higher amount of output data (n-numbers), for example to validate smaller effects in the sometimes highly variable zebrafish larvae population. For this kind of research, the development of an efficient HTP *in vivo* screening system for fluorescent zebrafish larvae is a great improvement to achieve a larger amount of data faster. The parameters to be analyzed in the developing fish larvae could be cell number in certain regions or organs as well as cell migration.

For this application note, a fast protocol to place one single green fluorescent zebrafish larva in a suitable position for imaging in each well of a 96-well plate was developed. HTP 96 full-length larvae images of a Tg(*Olig2:eGFP*) oligodendrocyte precursor cell (OPC) reporter line were obtained using the imaging technology of the EnSight multimode plate reader. This line shows Green Fluorescent Protein (GFP) expression only in the population of cells in which *Olig2* is expressed, marking OPC with green fluorescence.⁴ The algorithm “ZF-MigratingCells.kala” in the Kaleido software, which automatically detects the amount of dorsally-migrating OPC within the spinal cord on the images obtained by the EnSight, was validated. The efficacy of the algorithm “ZF-MigratingCells.kala” is shown in detail by comparing its automatic cell counting to traditional manual counting, analyzing the amount of OPCs in drug-treated and control larvae. It was confirmed that the algorithm displays the same statistical difference between non-treated, control, and drug-treated populations as manual counting.

Furthermore, the drug effects were validated using the time-lapse imaging technology of the EnSight, measuring the amount of migrated OPCs every two hours, and obtaining a trend of OPC migration in the control and treated population from 50 to 74 hpf.

Materials and Methods

Zebrafish, Eggs and Larvae Maintenance

Zebrafish (*Danio rerio*) were bred, raised, and maintained according to standard protocols.⁵ Adult fish are kept 14 h light on and 10 h light off at 28.0 °C in reverse osmosis water salted with Instant Ocean® to 500 µS and buffered with hydrogen carbonate to reach a pH of 7.4.

Adult fish were fed twice a day with fresh *Artemiae Salinae* and dried pellet food RM300. The fish were paired in breeding tanks by putting one male and one female, or one male and two females, dividing the two sexes overnight by a divider. Dividers were pulled just before onset of the light and fertilized eggs were collected 30 minutes after. Embryonal and larvae fish were kept in 30% Danieau water (15 mM NaCl, 0.5 mM KCl, 1 mM CaCl₂, 1 mM MgSO₄, 0.15 mM KH₂PO₄, 0.05 mM NH₂PO₄, 0.7 mM NaHCO₃) in a Petri dish at 28.0 °C, using Danieau water with methylene blue to avoid mold formation for the first 24 hours. To prevent pigmentation, larvae zebrafish were raised in Danieau with 0.003% N-Phenylthiourea (PTU Danieau) from 24 hours till 5 dpf, thus larvae stay transparent for the imaging analysis.

Drug Treatment

Drug and control solutions were pressure-injected through a pulled glass capillary in the yolk of 1-2 cell stage zebrafish eggs. The injection solution was calibrated in a drop of mineral oil on a microscope calibration slide. The injected sphere has a diameter of 135 to 140 µm with a drug amount of 2.75 ng. Other drugs could be given to the fish, for example by water immersion, if applicable.

Imaging

All imaging was performed with the imaging technology of EnSight. Seven images were made for each well: six different focus plane images for the fluorescent GFP detection and one image in the bright-field mode. In total, 672 images were obtained and analyzed from 96-wells in less than six minutes.

Plate Preparation for Larvae Imaging

A single preselected fluorescent zebrafish larva was placed in each well of a 96-well plate from Cellvis (Part No. P96-1.5H-N) applying the following protocol:

1. PTU Danieau from the larvae petri-dish was changed twice to wash away chorion residues and eventual debris that may interfere with larvae imaging.
2. A tip for P1000 pipet was cut on its top by 1 or 2 mm to widen the diameter, avoiding potential larvae damage in the transferring step. This tip was used in a P1000 set at 100 µL.
3. The P1000 with the previously cut tip was used to place larvae one by one from the Petri dish to the wells of the plate.
4. To prevent larvae movement during the analysis, MS222 (Tricaine methanesulfonate) was added to each well to reach 1x concentration.
5. Facultative: To avoid any external contamination (dust, debris, etc.), it is suggested to work under a fume hood.

Poly-D-Lysine coated plates were used for 5 dpf larvae. Lyophilized Poly-D-Lysine were ordered from Sigma-Aldrich (P6407-10X5MG, mol wt 70.000-150.000).

Protocol to Place Larvae in Lateral Position

1. A fine long plastic tip for gel loading was used to position each larva in the middle of the well (larvae should not stick on the border of the well) (Fig. 2): The EnSight imaging protocol has a cutoff at the upper, lower and left part of the wells, so larvae placed at those sides of the wells would be cut off from the images.
2. The plate was centrifuged at 16 g for five seconds, with maximum acceleration and deceleration. After this, most larvae were lying on the side position on the bottom of the well. Wells were visually checked to ensure the correct position: black eyes of the larvae should be overlapping (on top of each other) and therefore only one larvae's eye (the upper one) was visible from the top.

A schematic view of the plate preparation steps are resumed in Fig. 3.

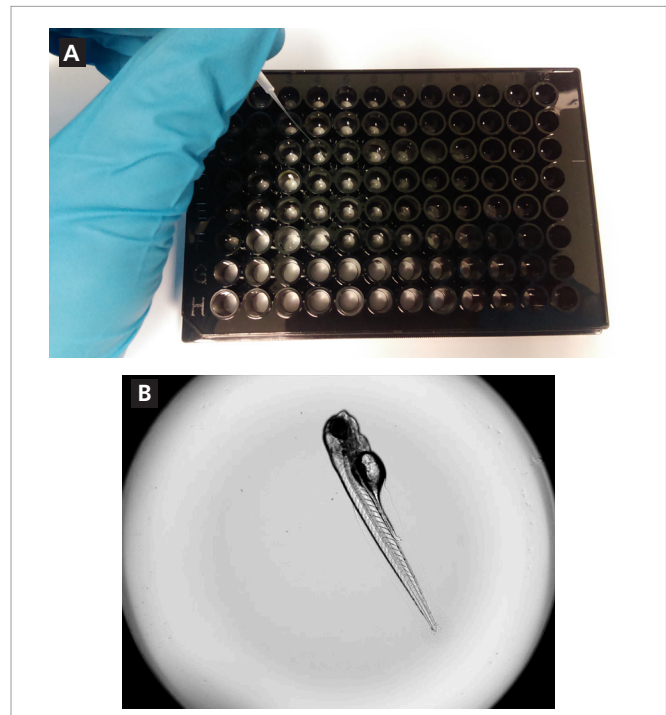


Figure 2. Positioning larvae in the 96-well plate. (A) Soft plastic needle is used to place larvae in the middle of the well. (B) Brightfield of larvae after the position and centrifuge.

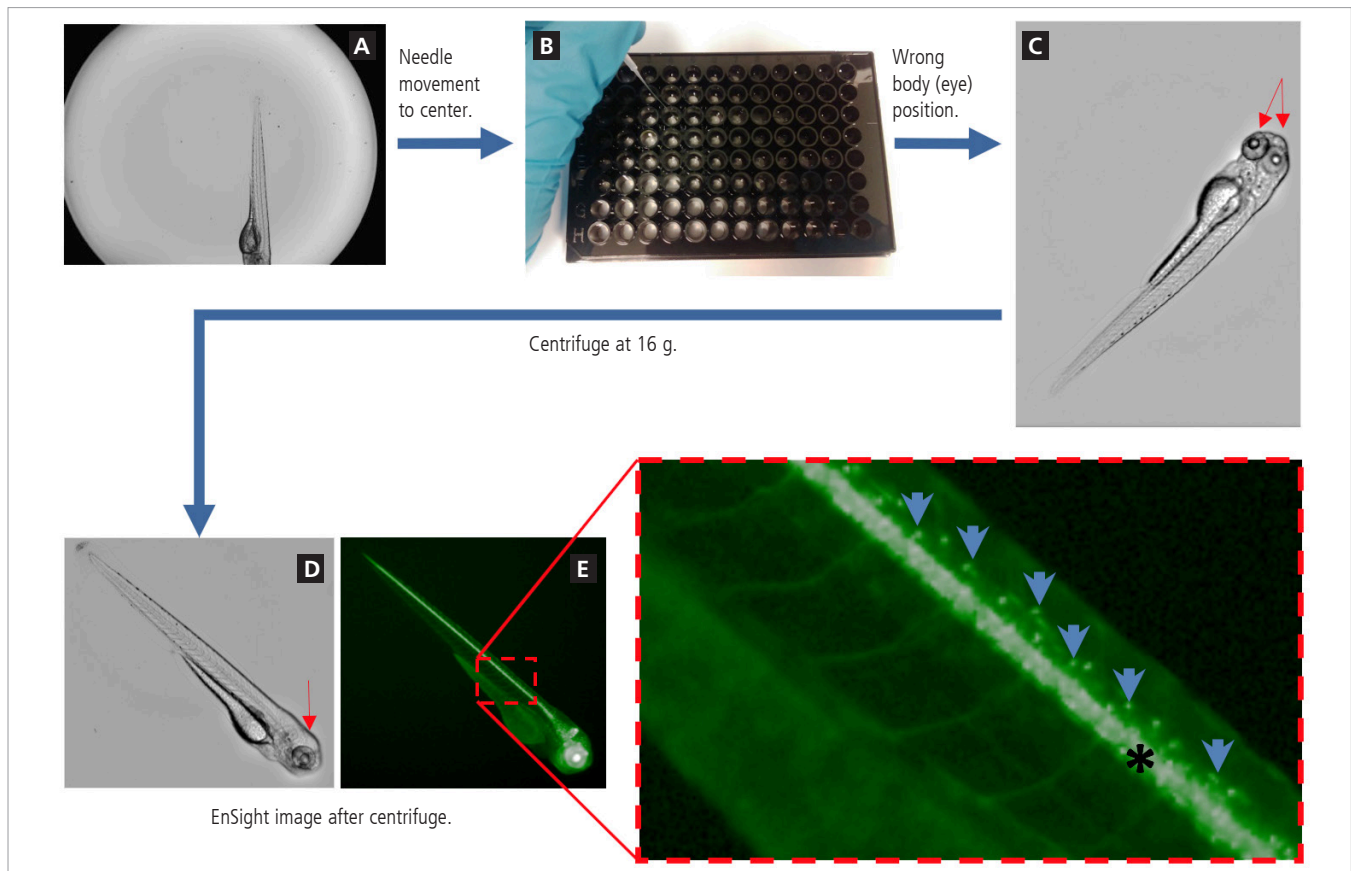


Figure 3. Positioning larvae in the 96-well plate. (A) Some larvae sticking to the border of the well. (B) A plastic (loading) tip places larvae in the middle of each well. (C) Some larvae lay on the belly (both eyes are detectable, black arrows), leading to a wrong detection of dorsally migrating cells. (D) After centrifugation for five seconds at 16 g, larvae are lying on the lateral side (only the upper eye can be seen, red arrow). (E) Fluorescence image of *Tg(olig2:eGFP)* larvae fish displaying the correct detection of dorsally migrating OPCs (blue arrow) which do not overlap with the fluorescence of the spinal cord (black asterisk).

Exposure Settings for Full-Length Larvae Identification

Zebrafish larvae are thicker than plated cells. More precisely, the fish slopes up from the cranial to its caudal region. To count the migrated OPCs through the whole length of the spinal cord, it is necessary to have the full body of a zebrafish larva in focus. In this study, six foci plane offsets in a range from 0 to 250 μm are enough to have the whole larva in focus (Fig. 4.). A bright-field image is necessary for the “ZF-MigratingCells.kala” algorithm to identify the presence of a single larva in each well by reading pixel texture values. All the foci parameters for the well images are resumed in Table 1. The algorithm automatically creates a stack of all these images using the best focused migrated cells.

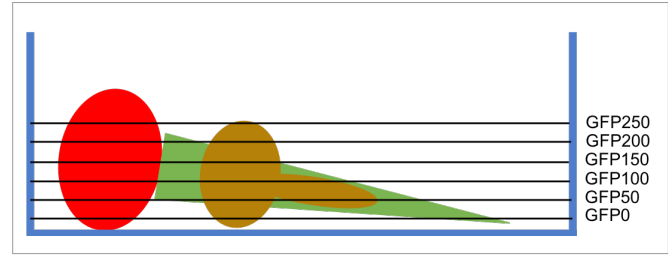


Figure 4. **Schematic lateral representation of a zebrafish larva in a well.** Six focus offsets are used for a full-length identification of a larva: head (red), yolk (brown), tilts/slopes and the body (green) relative to the bottom of the well. Schematic representations of layers of focus detection are shown with black lines.

Table 1. Details of imaging parameters for each focus height.

Name of the Channel	Excitation Wavelength [nm]	Excitation Power [%]	Exposure Time [ms]	Additional Focus Offset [μm]	Global Focus Height [μm]	Real Focus Offset for Channel [μm]
GFP0	465	100	50	0	+25	25
GFP50	465	100	50	50	+25	75
GFP100	465	100	50	100	+25	125
GFP150	465	100	50	150	+25	175
GFP200	465	100	50	200	+25	225
GFP250	465	100	50	250	+25	275
TRANSMISSION	735	4	4	0	+25	25

Plate Analysis

The 96-well plate was loaded with 32 non-treated, 32 control and 32 drug-treated larvae at the stadium of 50 hpf. The plate was placed into the EnSight and full-plate imaging performed every two hours up to 74 hpf to obtain a trend of the migration in a 24 h time window.

The measurements are repeated for larvae at 4 and 5 dpf to validate the effect of the drug (this application note only shows data for the batch at 4 dpf).

Results

Automatic Algorithm Counting Shows Same Trend as Manual Counting

The manual counting (MC) provides a decrease in the number of migrated OPCs in the drug treated group when compared to the control population with a significance p-value (t-test; $p < 0.0001$). The percentage of decrease is calculated with 35.1%. Different detection thresholds were tested to count the OPCs with the automated counting (AC), where the one fixed at 0.2 shows the most comparable results to the MC. This also gave a p-value significance of four stars between the control and drug-treated group and a percentage of decrease calculated to be 33.4% (Fig. 5).

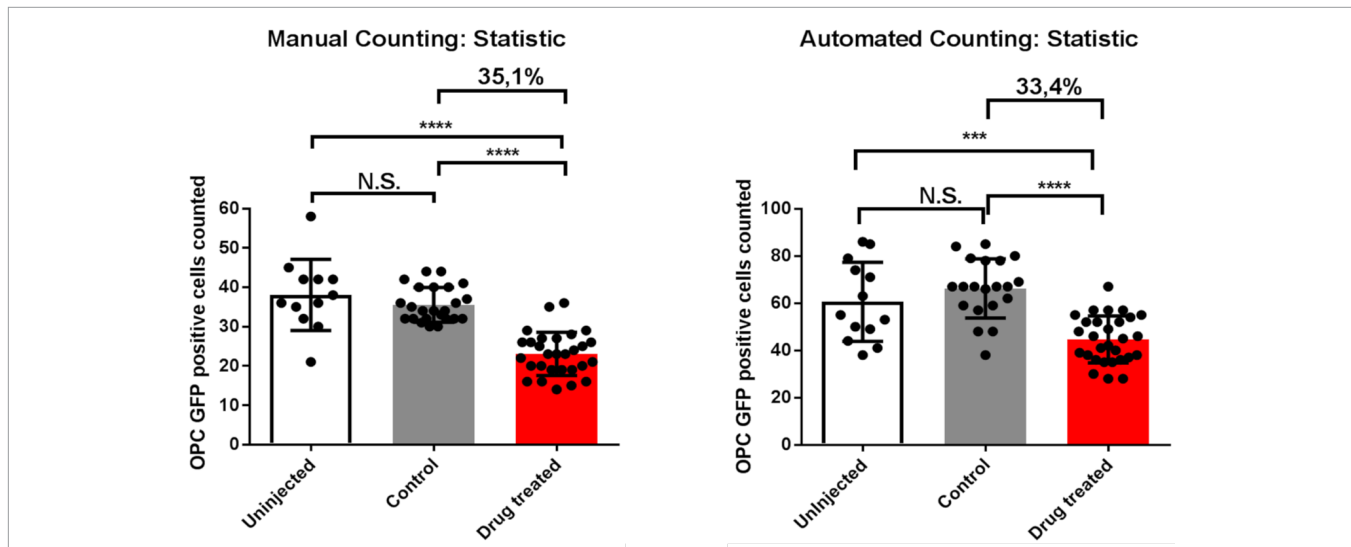


Figure 5. **Automated “Algorithm” Counting shows very similar statistics to Manual Counting.** Statistics of MC (left graph) and AC (right graph) of migrated GFP positive cells in larvae Tg(Olig2:eGFP) zebrafish at 4 dpf. Total numbers of cells are different since the MC counts only in a specific smaller region, while the AC detects cells migrating dorsally along the whole spine length. Despite this, AC with a threshold of 0.2 shows the same p-value between control and drug-treated and a very close value of percentage of decrease between these two populations.

Software Output

“ZF-MigratingCells.kala” assay specific analysis method displays for each larva the following parameters:

Table 2. Optional output parameters of “ZF-MigratingCell.kala” version 1.0.

Output Parameter	Meaning
Fish with Fluorescent Spine	Number of found fish larvae with a fluorescent spine in case more than one larva is accidentally placed in a well.
Length of Fish [μm]	Length of the larvae. If the larva is bent this refers to the shortest possible connection between the end points within the fish. Depends from the input “Contrast Threshold to identify Fish Larvae in Brightfield”.
Area of Fish [mm^2]	Area of the fish, measured in mm^2 .
Length of Spine [μm]	Length of what is identified as spinal cord. Depends from the input: “Spine Detection: Length of Orientation Filter”
Spine Fluorescence Intensity	Average of fluorescence intensity calculated for all the detected spine.
Min Distance of Spine to Fish Border [μm]	The spine should be located within the fish. Small values of this parameter may indicate non-ideal detection of zf outline or spine. Depends from the input “Contrast Threshold to identify Fish Larvae in Brightfield”.
Distance of Spine Center to Spine Border [μm]	Mass center of the spine should be within the spine. Negative values of this result indicate a mass center outside of the spine. This typically indicates a bent spine. If GFP expression of the spine is very low, or the fish is not in a proper position (e.g. lying on the belly rather than the side), the spine may be invisible. In this case, yolk autofluorescence can result in misidentification which also tends to yield bent regions and thus show negative values in this parameter.
Max Number of Migrated Cells per 100 μm	Highest cell density found in a sliding window over the cell search region. The cell density is normalized to a standard length of 100 μm .
Migrated Cells	Number of detected cells (of sufficient contrast) in the cell search region.
Contrast of Migrated Cells	Average contrast of the found cells.
Brightfield Texture in Potential Objects	Contrast of the object regions in Brightfield. This is determined prior to limiting the found objects to valid zf larvae (with fluorescent spine). Relatively low values of this result may indicate improper focus position, which is usually a result of the fish lying not properly on the plate bottom (e.g. due to improper positioning).
Brightfield Texture in Background	Texture in the regions without (potential) objects, mostly used during parameter optimization to set proper threshold values. The parameter is identical to the ‘Background Roughness’ of the task ‘region analysis’ in the standard methods.
Analysis Quality	String value that indicates noted issues (if not good). Particularly suspect results of the well detection are marked here.

For this application note, “Migrated Cells” and “Length of Fish” were chosen as parameters of interest.

Parameters such as “Length of Spine”, “Spine Fluorescence Intensity”, and “Min Distance of Spine to Fish Border” are used as quality control to detect correct positioned larvae and non-cut image, even if it is possible to use for different purposes.

Detection Algorithm Correctly Distinguishes Whole Larvae and Single Cells

The algorithm “ZF-MigratingCells.kala” combined with the imaging technology of the EnSight is able to recognize the body shape of a larva by calculating values of pixels texture. After this, the highly GFP fluorescent spinal cord is identified. The upper region for the migration area of the cells is defined dorsally of the spinal cord. In this later region, cells are identified and counted using threshold values (Fig. 6).

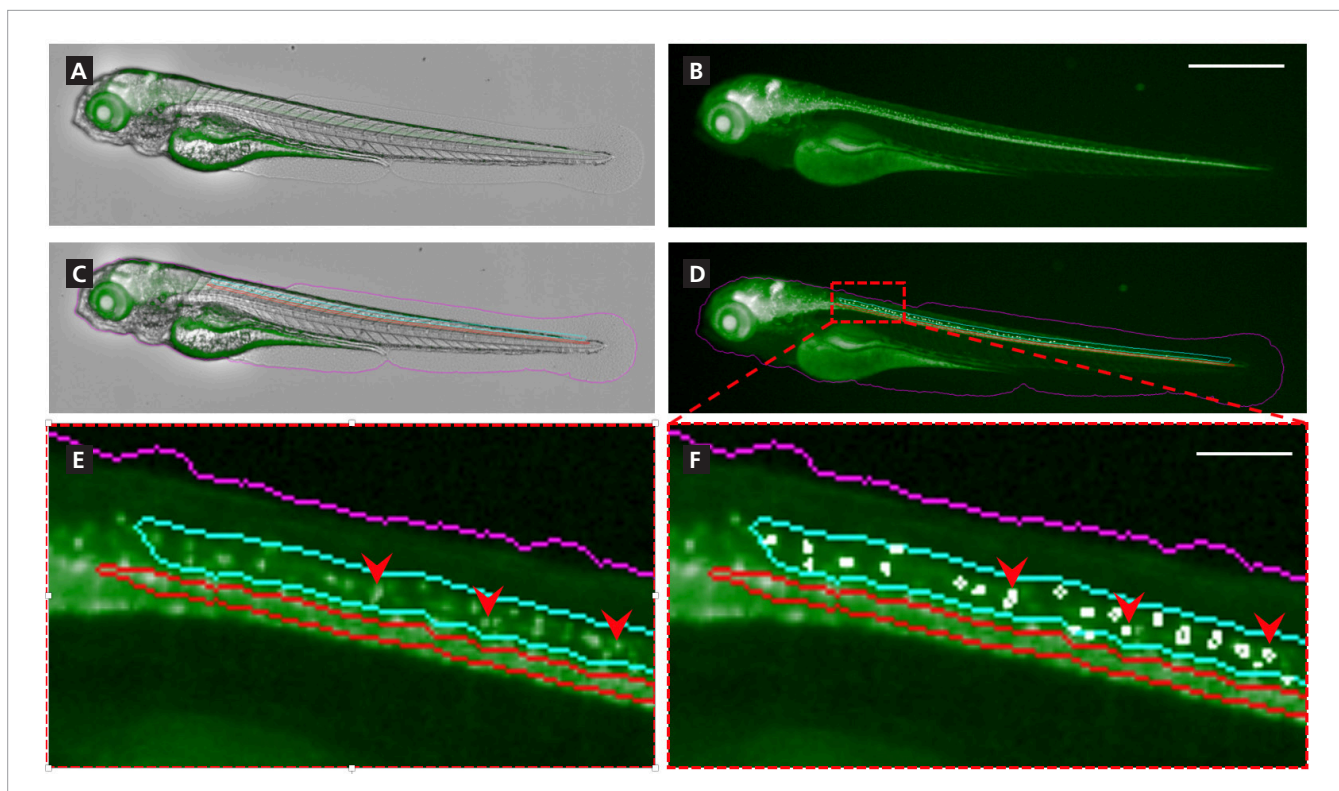


Figure 6. Tg(Olig2:eGFP) larva at 4 dpf analyzed by Kaleido software with the “ZF-MigratingCells.kala” algorithm. (A) GFP-positive larva displayed in Kaleido software with Brightfield + GFP 200 channel. (B) GFP 200 channel only. (C) Brightfield + GFP 200 channel. Larva is analyzed with detection algorithm, which allows one to identify the “Fish Border” (purple), spinal cord (red), region of migrated cells (light blue) and migrated cells (white dots). (D) GFP channel only with previously described parameters. (E) Detail of figure D displaying “Fish border” (purple), spinal cord (red) and region of migrated cell (light blue). Red arrows indicate some of the migrated cells. (F) Same parameter as picture D and E, in which detected cells are marked by white dots and counted. Images are made with EnSight, displayed in Kaleido software and analyzed with the “ZF-MigratingCells.kala” algorithm. Scale bar is 400 μm for images A to D and 50 μm for images E and F.

Algorithm Detects Difference Between Populations

Using the AC, this experiment shows that in a 24 h time-frame the drug-treated population constantly displays a lower amount of OPCs compared to the control and un-injected population (Fig. 7). Interpolations confirm that un-injected and control populations have the same gaining slope (p value > 0.05) while the drug-treated slope is statistically different from the controls (slope p value < 0.0001).

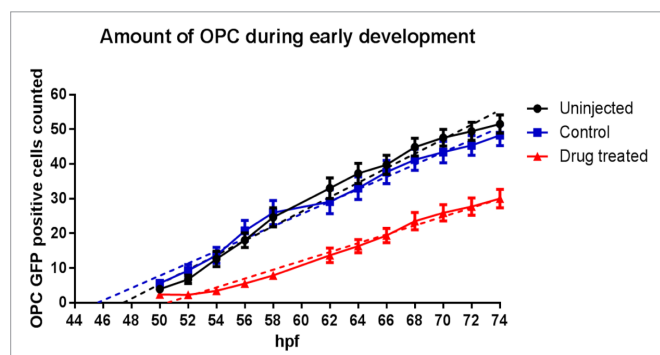


Figure 7. Treatment decreases the amount of migrating GFP positive cells. Data are mean \pm SEM, no statistical difference is detected at 50 hpf within the three populations (p value > 0.05). From 52 to 74 hpf, un-injected and control populations show no statistical difference (p value > 0.05) while differences are clearly detected between the drug treated population and control or un-injected population (P value < 0.0001).

Time-Frame Data Reveals Rate of Cells Per Hour and Start-Time of OPCs Migration

Interpolation lines ($y=mx+q$) of the data give information on the amount of migrating cells per hour and the initial time when OPCs start to migrate. The first value is extrapolated from the slope of the lines (m), the latter from the x intercept when $y=0$.

A delay in the migration of OPCs in the drug-treated population compared to the controls was confirmed.

Algorithm Detects Growth of Larvae

To test whether the effect of reduced numbers of migrating OPCs is due to a specific drug effect on myelination and not a general/unspecific effect (e.g. of the whole larvae development), the average body length of larvae for each population was plotted using the “Length of Fish” output parameter of the algorithm.

The algorithm is able to automatically measure the body-length (μm) of each larva and by plotting these values, it is possible to recognize whether the drug is interfering with the general growth (and therefore development) of the zebrafish larvae or not. As shown in Fig. 8, there is no difference in the length of the larvae within the populations (p value > 0.05) and all the larvae are growing similarly over time.

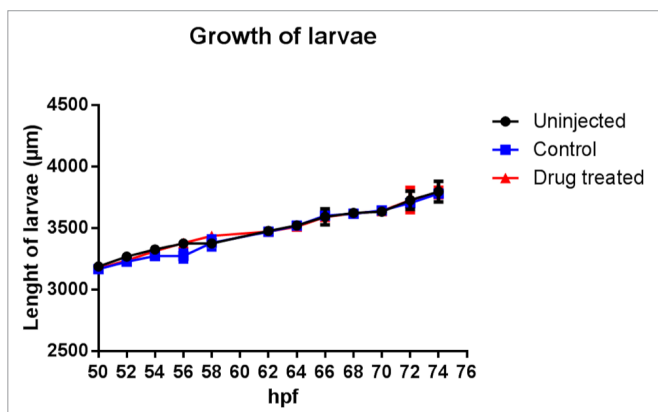


Figure 8. Body length of zebrafish larvae from 50 to 76 hpf in three different populations. Data are mean \pm SEM. No statistical differences were detected within all three populations.

Throughput Comparison

Three time-common steps within EnSight, 2-photon microscope (2-P), confocal microscope (CM) and fluorescent microscope (FM), were compared to confirm throughput and the total number of larvae that each instrument is able to analyze in one working day (n). The time-common steps are reported as following:

- Mounting: time (minutes) that occurs to firstly place zebrafish larvae on a support and finally in the correct orientation for the required imaging method (EnSight, 2-photon, confocal and fluorescence microscope).
- Imaging: time (minutes) that occurs for the machine to obtain the picture of the larvae.
- Analysis: time (minutes) needed to analyze the parameter of interest. Using the EnSight, parameters are counted automatically during the measurement; for 2-P, CM and FM, cells are counted manually in image.

These parameters were added together to obtain the total amount of time that is needed to obtain a result (number of migrated OPC). Since (n) and time are different for each method, (n) was used to calculate the time needed to have the result for a single larva (min/zf) and the reverse function (number of larva that are analyzed in one minute, zf/min). These two normalized ratios are used as an index to have an overview on the throughput of the EnSight technology.

Detailed parameters of each measurement method are reported in Table 3.

EnSight requires 1/2 minute (30 seconds) to have a complete image of the larva with annexed analysis, where the time is dominated by the sample preparation. 5, 35 and 10 minutes are needed to obtain the same results for 2-P, CM and FM respectively. In addition, these last techniques do not give whole-larvae analysis (unless it is at the expense of time). This means that to achieve the same EnSight (n), 8, 56, and 16 hours are required respectively for 2-P, CM and FM.

It is possible to mount more than one 96-well plate (e.g. 3), reaching to a (n)=288 with timing of \approx 90 min (normalized data for this (n) are not shown).

Looking at the ratio of zf/min in Table 3, the EnSight shows a ratio 10 times higher throughput compared to the 2-P, and 67 and 20 times the one of CM and FM respectively (Fig. 9).

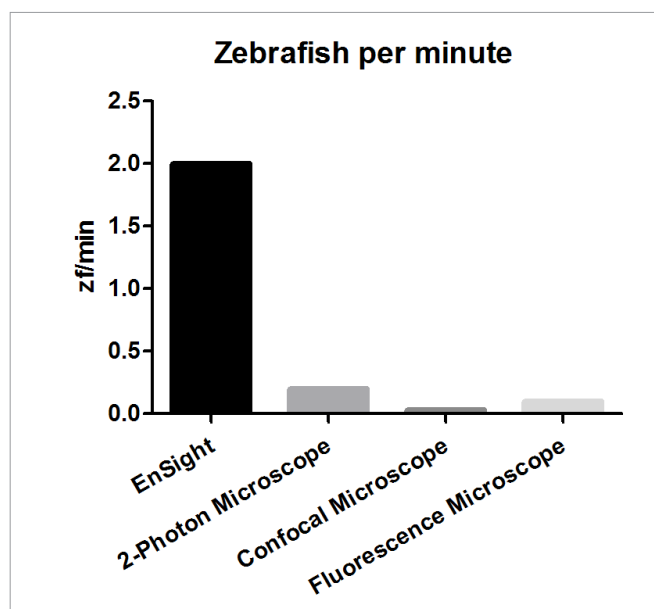


Figure 9. Amount of zebrafish larvae plated, imaged and analyzed in one minute (zf/min) by EnSight, 2-P, CM and FM. EnSight analyzes two zf/min 2-P, CM and FM analyze 0.2, 0.03 and 0.1 zf/min respectively.

Table 3. Comparison between different zebrafish imaging/analysis techniques. *Minutes are estimated by timing each step from trained users. ** 2-P data refer to the time need to image a specific spinal cord region and not the whole larva. ***CM data are timed for three zebrafish and just for half of the larvae length. Time can change depending on the area to image and on the model of confocal. EnSight imaging refers to the time needed for the plate reader to acquire and analyze seven pictures per well, giving a total of 672 images in six minutes. Analysis: except for EnSight imaging, all other techniques were analyzed manually using ImageJ.

	Number of Larvae (n)	Mounting (min)	Imaging (min)	Analysis (min)	Total Time (min)	min/zf	zf/min
EnSight	96	40		6	\approx 46(*)	1/2	2
2-P(**)	24	30	60	30	\approx 120(*)	5	0.2
CM(***)	3	15	90	15	\approx 120(*)	35	0.03
FM	15	20	60	30	\approx 110(*)	10	0.1

Conclusions

The proposed handling protocol is suitable to position larvae in the correct orientation for GFP positive cell detection and migration analysis.

The EnSight achieves HTP imaging of zebrafish larvae, producing seven images of a 96-well plate (larvae fish) in less than six minutes, including the algorithm analysis.

The detection algorithm is able to automatically identify and count the migrated GFP positive cells in the *Tg(Olig2:eGFP)* zebrafish line when larvae are properly positioned in a 96-well plate. This leads to a great amount of high quality data in only a few minutes of analysis. It also enables investigation of the time development of the GFP positive cells, as well as the growth of the zebrafish larvae. Since data is obtained from a high number of larvae, it is possible to gather more robust statistics within different populations. This screening method can certainly facilitate the identification of new drug candidates or compounds influencing cell numbers and/or *in vivo* cell migration.

The results also show that the drug used in this study was specific only to the GFP positive cells of interest for this study since no drug effect could be detected on the general development and growth of the zebrafish larvae.

Acknowledgement

Many thanks to the Appel laboratory for the *Tg(Olig2:eGFP)* zebrafish line.

References

1. An, F. W., & Tolliday, N. (2010). Cell-based assays for high-throughput screening. *Molecular biotechnology*, 2(45), 180-186. doi:10.1007/s12033-010-9251-z.
2. Giacomotto, J., & Ségalat, L. (2010). High-throughput screening and small animal models, where are we? *British Journal of Pharmacology*, 2(160), 204-216 doi:10.1111/j.1476-5381.2010.00725.x.
3. Buckley, C. E., Marguerie, A., Roach, A. G., Goldsmith, P., Fleming, A., Alderton, W. K., & Franklin, R. J. (2010). Drug reprofiling using zebrafish identifies novel compounds with potential pro-myelination effects. *Neuropharmacology*, 3(59), 149-159. doi:10.1016/j.neuropharm.2010.04.014.
4. Shin, J., Park, H.-C., Topczewska, J. M., Mawdsley, D. L., & Appel, B. (2003). Neural cell fate analysis in zebrafish using olig2 BAC transgenics. *Methods in Cell science: An Official Journal of the Society for In Vitro Biology*, 1-2(25), 7-14. doi:10.1023/B:MICS.0000006847.09037.3a.
5. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 3(203), 253-310. doi:10.1002/aja.1002030302.
6. Lawrence, C. (2007). The husbandry of zebrafish (*Danio rerio*). A review. *Aquaculture*, 1-4(269), 1-20. doi:10.1016/j.aquaculture.2007.04.077.