Article

ZFTool: A software for automatic quantification of cancer cell mass evolution in zebrafish

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Abstract: Background: Zebrafish (*Danio rerio*) is a model organism emerged for the study of human cancer. Compared with the murine model, the zebrafish model has several properties ideal for personalized therapies. The transparency of the zebrafish embryos and the development of the pigment-deficient "casper" zebrafish line give the capacity to directly observe cancer formation and progression in the living animal. Automatic quantification of cellular proliferation in vivo is critical to the development of personalized medicine. **Methods:** A new methodology was defined to automatically quantify the cancer cellular evolution. ZFTool was developed to establish a base threshold that eliminates the embryo auto-fluorescence and automatically measures the area and intensity of GFP (green-fluorescent protein) marked cells and define a proliferation index. **Results:** Proliferation index automatically computed on different targets demonstrates the efficiency of ZFTool to provide a good automatic quantification of cancer cell evolution and dissemination. **Conclusion:** Our results demonstrate that ZFTool is a reliable tool for the automatic quantification of the proliferation index, being a measure of cancer mass evolution in zebrafish eliminating the influence of its autofluorescence.

Keywords: xenotransplant; cancer cells; zebrafish image analysis; in vivo assay

1. Introduction

Over the past 15 years, the zebrafish (*Danio rerio*) has emerged as a model system for the study of human cancer. The transparency of the zebrafish embryos and the development of the pigment-deficient "casper" zebrafish line allow the scientists to observe cancer formation and progression directly in the living animal. The optical clarity of zebrafish can be exploited further by the use of fluorescent tags to label specific cell lineages to visualize tumor processes including initiation, progression, and regression. The zebrafish is experimentally amenable to transplantation assays that test the serial passage and malignant potential of fluorescently-labeled tumor cells as well as their capacity to disseminate and/or metastasize. Due to its fecundity and the optical clarity during embryonic development, the zebrafish has proven to be an excellent in vivo model system for high throughput drug screening, because it allows the visual assessment of both drug efficacy and toxicity [1]. During last years the improvement of xenotransplantation of human cancer cells into zebrafish embryos has emerged as a powerful tool complementary to murine models [2]. Mikut et al. [3] described the state of the art for automated processing of zebrafish imaging data and they identify future challenges for zebrafish image analysis community. The zebrafish characteristics are exploited to address important questions in genetics, developmental biology, drug discovery, toxicology, and biomedical research. Zebrafish models exist for a broad range of human diseases, as cardiovascular diseases [4], cancer [5], or movement disorders [6]. The present paper describes our research about measuring evolution on cancer cells on zebrafish. Although several articles have been published on zebrafish xenotransplantation [7–10], our aim is to optimize this technique for primary

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cultures originated from colorectal cancer patients in 48h zebrafish embryos. Previously, RT qPCR and 2D imaging have been used to quantify both proliferation and migration. However, the techniques described so far do not provide an accurate measurement for both parameters. We propose accurate quantification on zebrafish embryos by temporal analysis of xenotransplanted cells marked with GFP (green-fluorescent protein). In the literature, some other works do research on measuring the evolution of cancer cells, and some of them developed an image analysis tool for it, like ZebIAT[11], use the Fiji distribution of the free software ImageJ, pioneer in bioimage analysis [12] or use commercial software [13]. A good revision of software for zebrafish image processing is in [3]. Nevertheless, these software tools are too specific, and none of them perform the analysis objective of our research, so we had to develop our own methodology.

2. Materials and Methods

2.1. Material

The images used in this paper were captured from zebrafish embryos as described in the previous study for which this software was initially developed [14]. In the following, we resume those conditions.

Zebrafish embryos were obtained from mating adults according to standard procedures. The human colorectal cancer cell line HCT116 was obtained from American Type Culture Collection (ATCC, Catalog No. CCL-247) and cultured using McCoy's 5A Medium containing 10% FBS (GIBCO, Invitrogen) and 1% Pen/Strep (GIBCO, Invitrogen) at 37ºC with 5% CO2 in a humidified atmosphere. The HCT116 cell line was transfected to express GFP constitutively. HCT116 cells were transduced using a lentiviral-driven GFP construct (Sigma, Mission TurboGFP, SHC003 V). Cells were placed 72h post infection under selective pressure using 10 μg/ml puromycin.

Two days post fertilization (dpf), zebrafish embryos were dechorionated (if needed) and anesthetized with 0.003% tricaine (Sigma). Cell injections were performed manually right into the yolk of the embryo. Incorrectly injected embryos without cells inside of the yolk, or showing them in the circulation after xenotransplantation were discarded. After injection, 2dpf embryos were incubated at 36C in 24-well plates with salt dechlorinate tap water (SDTW, chlorine free water obtained with a reverse osmosis filter system) for 72h to check the proliferation of the cell line by ZFTool. Each embryo was photographed with AZ-100 Nikon fluorescence stereomicroscope at 0hpi (hours post injection) and 72hpi to be analyzed by ZFTool software.

Figure 1 shows a typical image of both zebrafish and GFP mass at 0hpi and 72hpi. The GFP image is overlaid over the original embryo image just for positioning it over the zebrafish.

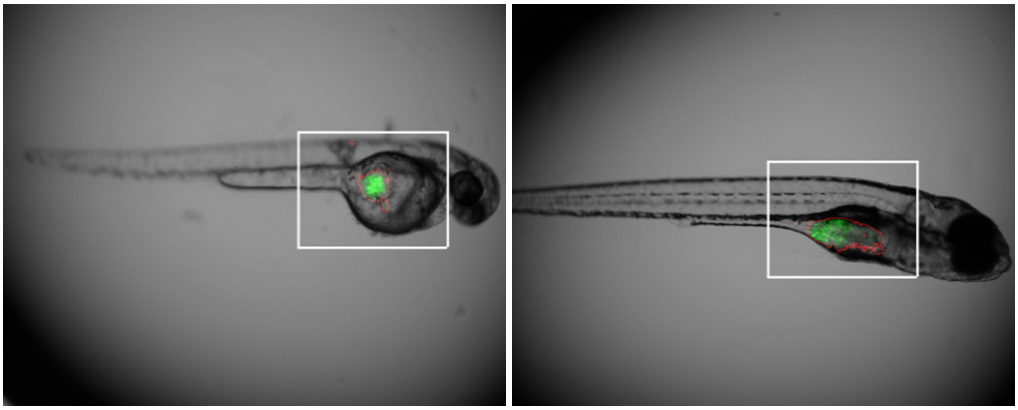

Figure 1. Example of segmentation over a characteristic image (zebrafish #8 at 0hpi and 72hpi) where the GFP value and the contour image are overlaid in green and red, respectively. The white rectangle will be the region of interest in next figures just to clearly observe the evolution of GFP area with different thresholds.

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2.2. Methods

The objective of ZFTool is to automatize and improve the task of measuring the number and mean value of GFP pixels to compare them for 0hpi and 72hpi in order to quantify the cancer mass evolution with time. ZFTool was developed as a Matlab toolbox and it is available at https://gitlab.citius.usc.es/zebrafish/zftool. The input to the system are two images at 0hpi and two images at 24, 48 or 72 hpi. The ouptut is both numerical (final threshold, mean areas and GFP intensities, proliferation index) and graphical (GFP intensity and area evolution, threshold and images with initial and final perimeter). As was stated before, zebrafish is ideal for quantification of GFP masses because of its transparency, but a problem arise caused by the variable autofluorescence of the fish, specially in the yolk area. In order to accurately quantify the GFP evolution, a pre-processing must be applied to eliminate the autofluorescence region from the count. This preprocessing is based in the observation of evolution of GFP area with a threshold from 0 (no threshold) to 50 in steps of 5 (see an example in Figure 2).

As was stated before, this autofluorescence is variable, depending of the fish and on the hpi, so this threshold must be adapted to each case. ZFTool software evaluated the graph of GFP threshold with respect to the area and select as threshold the point in which this area remains stable.

Once this autofluorescence is eliminated, some parameters are computed in order to measure the cancer mass evolution: the number of GFP pixels in the image (nGFP), which represents the area of the cells inside the yolk sac at two different times and the GFP intensity Medium Value (GMV), which represents the medium intensity of the fluorescence inside the yolk. By multiplying the nGFP number by the GMV of each image, we determined the proliferation ratio between 0hpi and 72hpi to estimate the cell growth. The result obtained at 72hpi was divided by that obtained at 0hpi, yielding a proliferation index value (PI):

$$PI = \frac{nGFP_{72hpi} * GMV_{72hpi}}{nGFP_{0hpi} * GMV_{0hpi}} \quad (1)$$

A PI value=1 means that cells remain stable during incubation, a PI slightly higher than 1 usually indicates a dissemination of cells (greater area, same intensity), a PI near or over 2 indicates a proliferation of cells (greater area, greater intensity) and a PI lower than 1 indicates tumor cell death.

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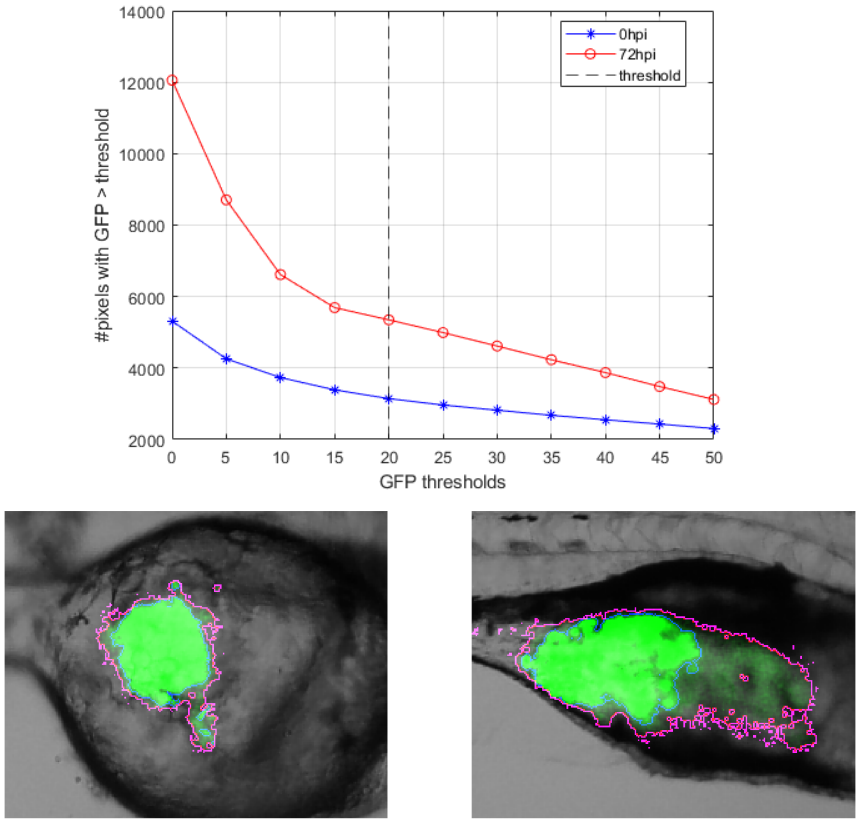

Figure 2. Example of autofluorescence in the yolk sac for fish #8 at 0hpi and 72hpi. The graph on the top shows the area evolution with respect to the GFP threshold in steps of 5. It can be observed the abrupt decay in the area caused by the fish autofluorescence. When the area remains stable for 3 iterations, the threshold is fixed. Down: threshold at 0hpi in magenta, and final threshold (20 in this case) in blue. The GFP (green channel) was artificially enhanced in order to make it visible the autofluorescence causing the initial contour. This is better seen in the image at 72hpi (right). The original images are shown in Figure 3, where the autofluorescence cannot be appreciated.

We must have in mind that, in order to compare these measures at 0hpi and at 72hpi, the threshold in both cases must be the same, so we will compute these two thresholds automatically and then the biggest one will be applied to both images.

Due to the zebrafish autofluorescence and the variability of capture conditions, the segmentation threshold will not be always the same. In order to design a methodology for automatic computation of this threshold, several tests were done with a training set of 14 zebrafishes applying thresholds from 0 to 50 in 5 intervals, discarding regions with area less than 10 pixels. Figure 3 shows the evolution of area and intensity for two characteristic zebrafishes(#8 and #14), one with no proliferation of cell mass and another one with proliferation of cancer cell mass.

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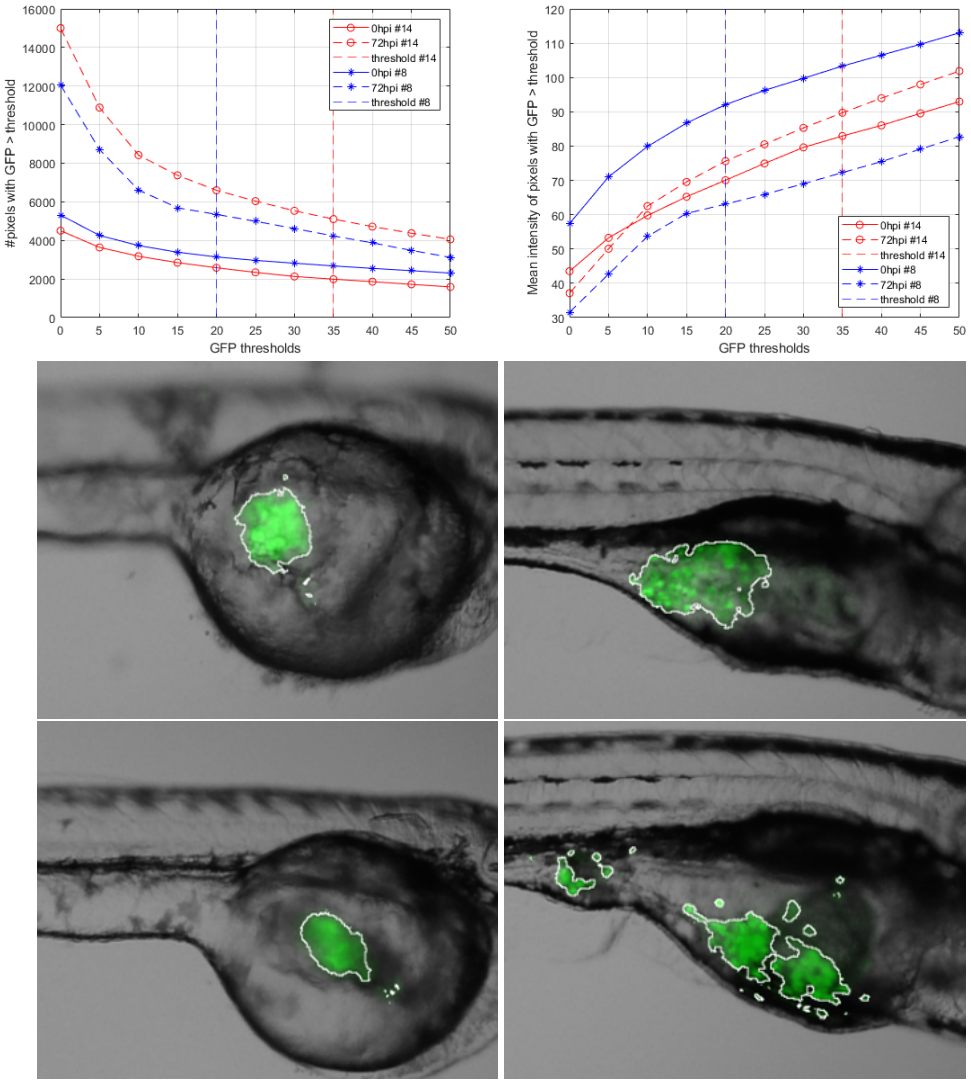

Figure 3. Characteristic zebrafishes #8 and #14. Up: Fish #8 (blue) presents greater area and lower intensity after 72h while fish #14 (red) presents greater area and higher intensity after 72h. Threshold final values for #8 and #14 zebrafishes are 20 and 35, respectively. Middle and bottom rows: Thresholded GFP regions for zebrafish 8 (middle) and zebrafish 14 (bottom) for 0hpi (left) and 72hpi (right). Final threshold for fish 8 is 20 and for fish 14 is 35. We can observe from left to right the dissemination of cancer cells in middle row and the proliferation in bottom row.

After applying the whole algorithm to the test set we can conclude that when the cancer cells disseminate over the fish, the GFP region area is greater but the mean GFP mean intensity gets lower for 72hpi than those values for 0hpi. On the other hand, when there is cell multiplication, both the GFP area and GFP mean intensity achieves greater values, depending on proliferation factor. This fact can be observed in Figure 3, where we can conclude dissemination of cells for fish #8 and proliferation for fish #14. For fish #8 the regions after applying the threshold can be seen in Figure 3 (middle), where it can be seen that the region is more irregular and the cells are disseminated over the region. For fish #14 we can observe in Figure 3 (bottom) a proliferation of cells, the GFP intensity is greater and the area is also greater.

3. Results

To prove the algorithm and assess its performance, we analyzed images belonging to a test set. This test set is composed by 18 zebrafishes with captures taken at 0hpi and 72hpi. Results confirmed the assumption made in previous section, that when area and intensity

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go to greater values, it is a symptom of proliferation and when the area is greater and intensity is lower, it is a symptom of dissemination of cancer cells. The applied automatic threshold performs well in all cases and agree with those concluded by an expert. Table 1 shows the measurements for zebrafishes #8 and #14 and the final proliferation index.

Table 1. Final thresholds and measurements of areas and mean green value for fishes #8 (dissemination, PI around 1) and #14 (proliferation, PI greater than 2).

Zebrafish #	Threshold	$nGFP_{0hpi}$	GMV_{0hpi}	$nGFP_{72hpi}$	GMV_{72hpi}	PI
#8	20	3144	92.08	5349	63.07	1.16
#14	35	1991	82.94	5106	89.72	2.77

4. Discussion and Conclusion

Some issues to discuss are related with the correlation with 2D and 3D analysis and the affection of autofluorescence.

Related to the first issue, although the relation between 2D and 3D measurements must be demonstrated, some previous experimentation made by the authors showed that there is a correlation between 2D and 3D, as the graphs of area and volume follow the same tendency as that in Figure 3 [15]. With respect to the second issue, as ZFTool works directly with the channel green image, if another image (i.e., channel red) is provided, all the computations will work properly, as results do not depend on the channel being used. In case that the fish do not have autofluorescence in that channel, the graphs of evolution shown in Figures 2 and 3 will have an almost horizontal tendency, so the value of the threshold will be almost indifferent, and ZFTool is able to work in this case also.

Regarding the biomarkers used to label the injected cells, two different experimental approaches for the xenograft experiments can be carried out. On the one hand, transient libeling of the cells with a protein expressed in the cytoplasm like GFP, RFP or Mcherry, and on the other hand, labeling the cells with a lypophilic dye like DiI. In the last approach, ZFtool function as previously described, erasing the autofluorescence, being important in this case because working with a lypophilic dye could lead to an increase in autofluorescence or artifacts due to the lysis of the injected cells and the spread of the dye across areas of the zebrafish embryo. In this last case, it is not possible to calculate a proliferation ratio, but a more accurate fold change between the conditions tested against the control.

Another issue is if it could be adequate to fix the autofluorescence of the fish previously to the injection. We have decided not to do this as the autofluorescence is dependent of each individual, by the stage of development, and by the drugs under research. This is precisely why we fixed the threshold in this way: we computed the first possible threshold for each fish in each moment and fixed the greater value as the common threshold. As the graphs in Figures 2 and 3 show, this will be the first possible threshold, when the autofluorescence is eliminated, although selecting a subsequent threshold barely affected the mean area and intensity of the tumor.

In our research, a proliferation index was defined, as indicated in equation (1). This parameter by itself should be an indicator of proliferation in most of the experiments. Nevertheless, there are different scenarios for the proliferation index, considering that if the dissemination is larger than the proliferation, the increase in the proliferation index could be due exclusively to spread instead of division of the cells. For this reason ZFTool also offers as output both the intensity and area, and apart from that, the graphs and the images to be used as complementary diagnostic by the researchers. Although a nuclear marker could be used for quantifying cell proliferation, the quality of the images and its 2D character do not permit such exact counting, so we used the intensity of the GPF as an indicator of the number of superposed cells.

Once systematized, improved and tested, the aim of the whole procedure would be to conduct an in vivo assay screening that could help clinicians to decide the best chemotherapy combinations for each patient through the injection of patient cancer cells

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into the zebrafish embryos. The procedure would consist in transplanting tumor cells extracted from a patient in zebrafish embryos, incubate them at the desired temperature and assay a range of different drugs for such cancer. In just a few days, and after a careful process of image and statistical analysis, this methodology will provide enough information to help medical team decide, with a higher probability of success, which of the available drugs is more suitable to prevent the growth and metastasis of the cancer in that specific patient. In the oncology field, finding the right treatment from the first steps of diagnoses will greatly increase the probability of success, improving the quality of life and survival of the patient, as well as giving rise to a significant reduction in the cost of treatments. Although the objective could be achieved, there are many variables that this technique needs to take into consideration to evaluate the proliferation of the cancer cells and treatments, such as the microenvironment of the tumor and the matrix in the human body.

As a conclusion, in this work we have designed an algorithm to automatically perform the thresholding and computation of GFP area and mean intensity values. These values are characteristics as to demonstrate the evolution of the injection of cancer cells into the yolk sac of a zebrafish embryo. This computation is of great interest for cancer research as zebrafish allow in vivo assays and we can do a reliable, repeatable and quick computation of characteristic features. We have also defined a so-called proliferation index as a measure of the degree of dissemination or multiplication of tumor cells.

Author Contributions: Conceptualization, MJC and LS; methodology, MJC and NVB; software, MJC and NVB; validation, PCS and LS; investigation, MJC, NVB, PCS and LS; writing—original draft preparation, MJC and NVB; writing—review and editing, MJC, NVB, PCS and LS. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The care, use and treatment of zebrafish were performed in agreement with the Animal Care and Use Committee of the University of Santiago de Compostela and the standard protocols of Spain (Directive 2012-63-UE). The protocol was approved by the Animal Care and Use Committee of the University of Santiago de Compostela.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

5-FU	5-Fluorouracil
dpf	Days post-fertilization
GFP	Green-Fluorescent Protein
GMV	GFP medium value
hpf	Hours post-fecundation
hpi	Hours post-injection
nGFP	Number of GFP pixels

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