Optimizing image analysis to verify the accuracy of measurement of drug responses of patient derived xenografts in zebrafish embryos

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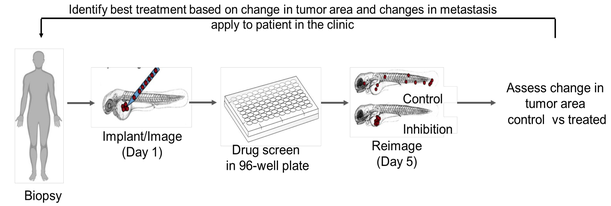
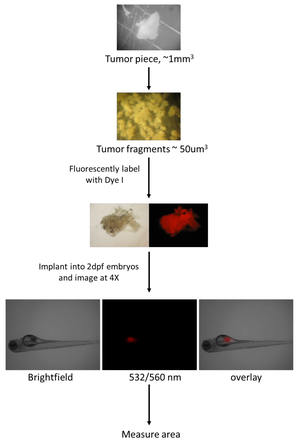
ABSTRACT

Zebrafish (Danio rerio) serve as a key organism for cancer research. Our lab has developed a patient derived xenograft model in zebrafish embryos, Zevatars, in which we can identify the best therapy for a patient's tumor in a personalized medicine setting. Tumor fragments labelled with CM-dil (a non-toxic, intracellular fluorescent dye) are prepared from a single 1mm^3 cube block of tumor tissue and implanted into 2dpf zebrafish embryos using tungsten needles. The implanted embryos are treated with drugs in 96 wells and imaged Day 1 of treatment and subsequently re-imaged at Day 3-5 at 4X magnification and 532/560 nm. Change in tumor size in the yolk is measured as change in area between Day 1 and Day 3-5. Others have devised high throughput imaging methods for drug screening in zebrafish but these have concentrated on examining changes in the characteristics of the zebrafish itself rather than the xenograft cells it is bearing. In this study we aimed to optimize image acquisition and analysis methods to enable high throughput and accurate assessment of tumor changes in Zevatars. We initially utilized two methods 1) Area measurements using 2D images from an epifluorescent microscope and a manual thresholding method using ImageJ. 2) Planar fluorescence tumor volume estimates measurements using pseudo Z-stacks from a Keyence fluorescence microscope using MATLAB. Our analysis revealed that there was no difference in tumor size when estimated from a 2D image compared to a pseudo Z -stack image. We then aimed to use multiple methods to validate our imaging analysis; 1) Direct cell counting by dissociating implanted embryos and counting dye I labelled cells. 2) Using whole mount immunohistochemistry to stain and count implanted human cells. 3) Using qPCR to estimate cell number by assaying for human target RNAse P in our implanted zebrafish. Although Zebrafish xenografts are becoming more widely used as a method to model cancer research, the imaging approach has not been extensively validated. Standardizing our imaging methodology allows us to apply Zevatars in the clinic for more accurate prediction of patient tumor response.

BACKGROUND





- Patient tumor pieces from biopsies are implanted into zebrafish which are subsequently treated and analyzed (Zevatars).
- The purpose of the Zevatar model is to create a high-throughput and low cost drug-screening model that can be used in a personalized medicine setting.
- Currently we use manual analysis with image J. Our goal is to develop a method to more efficiently validate the images using MATLAB in order to ensure that the analysis of each drug screen is reported both rapidly and accurately.
- Tumor biopsies are labeled with DiI and implanted into the yolk sacks of 2 dpf zebrafish embryos and imaged. Implanted embryos are then randomly assigned to treatment groups and reimaged 3-5 days post implantation to identify the most effective drug treatments. 2D Images are then analyzed according to the workflow shown in **Figure 4**.

Optimization of image analysis

Determination of focal layer of tumor

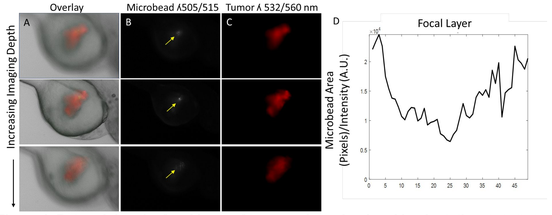


Figure 1. Determining the focal layer of tumor using microbead implantation. Labelled tumor fragment and a 2.5µm microbead (InSpeck™ green λ505/515 Molecular Probes) were implanted into the zebrafish simultaneously (the microbead is presumed to be centered in the fragment). Z-stack epifluorescent images were taken of the implanted zebrafish **(Figure 2A)**. The microbead was imaged at varying depths to determine the focal plane **(Figure 2B)**. The focal plane is indicated by the smallest microbead size, thus the focal plane was determined by dividing the microbead area by the microbead intensity at each layer of the z-stack. The image with the smallest microbead area and the highest intensity is shown as the trough in **Figure 2D**. The focal plane of the microbead and tumor are the same, thus the focal plane of the tumor is shown as the centre image in **Figure 2 panel C.**

Identification of tumor area of 2D image using threshold method

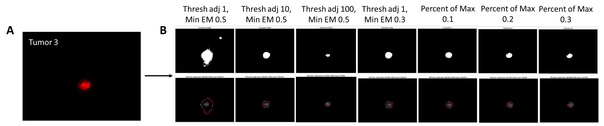
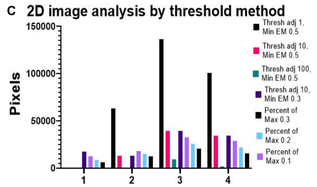


Figure 2. 2D image analysis. Two methods were initially used to analyze the tumor fragments such as the one shown in **Figure 3A.** The Otsu method was implemented by using the "otsuthresh" command in MATLAB (MathWorks, Natick, MA) and the percentage of maximum fluorescence (POM) method was implemented by taking the maximum fluorescence value in the image and multiplying it by a user's determined percentage. Multiple parameters were tested for each method **(Figure 2C)**. As shown in **Figure 2B**, the Otsu method with a multiplier of 10 (effectiveness metric 0.3) and the POM method using 20% of the maximum fluorescence produced similar values. Further analysis revealed that the POM method was more reliable.



Comparison of 2D analysis vs middle slice from z-stack

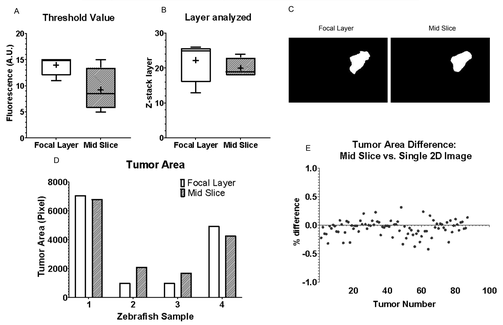


Figure 3. Comparing 2D image analysis to z-stack of image analysis. The focused layer (determined by the microbead analysis in **Figure 2**) was often located in the middle of the z-stack **(Figure 3A)**. **Figures 3C and 3D** demonstrate that while there is variation in the image of the tumor, the trend in the area of the fragment was consistent. In **Figure 3E**, single 2D images were analyzed and compared to the z-stack mid slice image analysis and the average difference was ±10.5%. This indicates that 2D images are sufficient to perform our analysis.

Application of coding logic

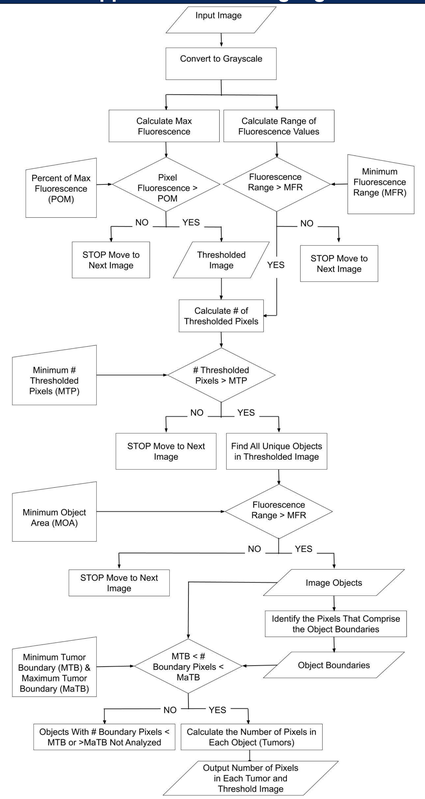


Figure 4. Workflow illustrating the code logic for image analysis

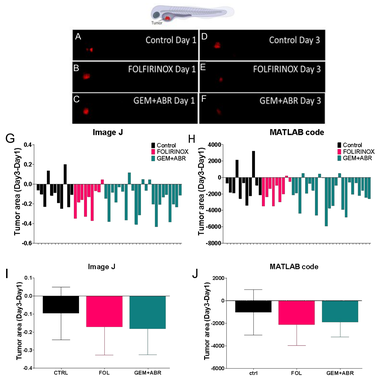


Figure 5. Analysis of Zevatar tumor implants reveals that both Image J and MATLAB analysis methods report the same trend in tumor response. Tumors were implanted into the yolk of the zebrafish 2 dpf. Tumor was stained with DiI. **A** serves as a control, the other implanted zebrafish were treated with folfirinox (FOL) (4.2mM FU, 1mM folinic acid, 0.08mM irinotecan, 0.08mM oxaliplatin) **(B)** or gemcitabine (GEM)+Abraxane (ABR) (0.1mM +.005mM) treatment **(C)**. **D,E,F** are images of the same tumors 3 days post treatment. **G** is a representation of the change in area of the tumor fragments between Day 1 and Day 3 in each embryo based on image J and manual thresholding analysis. **H** is a representation of the change in area of the tumor fragments between Day 1 and Day 3 in each embryo based on MATLAB analysis. **I** and **J** represent the average change in area between treatment groups.

Validation of imaging code

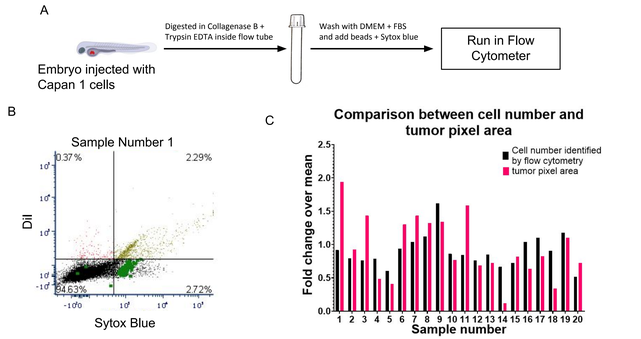


Figure 6. Cell numbers calculated from flow cytometry data compared to tumor pixel area calculated using optimized code: Individual Zebrafish embryos injected with capan 1 cells were digested by vigorously pipetting each embryo in a 150ul of dissociation mix (collagenase + trypsin EDTA) in individual flow tubes. Then, 150 ul of DMEM with FBS, 20ul of spherotech beads 14.7µM cat no – PPS-6, and 2 ul of Sytox blue stain were then added to each tube immediately prior to running each sample **(A)**. The cell number as quantified using flow cytometry **(B)** was then compared to the tumor pixel area from 2D images **(C)**. Data shows that in majority of samples tumor pixel area is similar to cell numbers calculated from flow cytometry

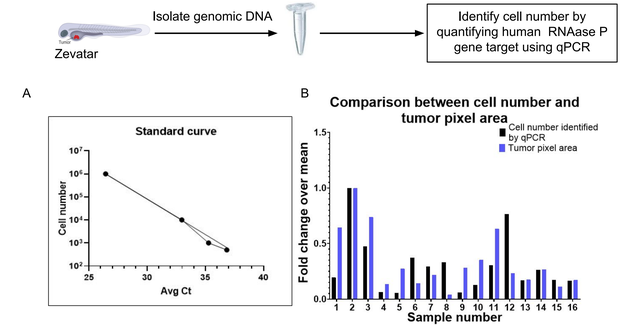


Figure 7. Cell numbers calculated from qPCR compared to tumor pixel area calculated using optimized code: A standard curve was generated by plotting the average Ct after human RNASe P amplification from known number of cells **(A)**. Cell numbers for samples was extrapolated from this curve and then compared to the tumor pixel area calculated from 2D image analysis **(C)**. Data shows that in majority of samples tumor pixel area is similar to cell numbers calculated from qPCR

Discussion and Future Directions

- Our results indicate that analysis of 2D images using MATLAB software can portray tumor size as accurately as z-stack image analysis. This is favorable as it allows for high-throughput imaging and analysis.
- Both the flow cytometry data and qPCR data present promising initial results, but both have several limitations that must be overcome in their current application.
- Flow cytometry: A primary variable in the analysis was the variation in bead number, due to the volume of samples and efficiency necessary to keep the cells alive, several samples had very low numbers of beads causing an overestimation of cell number. Additionally, the image analysis cant distinguish between live and dead cells which can also lead to an overestimation of cells.
- qPCR: Similar to the flow cytometry data, the image analysis cannot distinguish between live and dead cells leading to an overestimation of cell number. There is also a fair likelihood of loss when isolating genomic DNA due to the low percentage of human DNA which would lead to an underestimation of cell number.
- Future experiments should conduct further analysis using droplet digital PCR so we can get a direct readout of copy number without relying on a standard curve. We will also adapt our current flow cytometry protocol for tumor tissue in order to confirm the results reported using injected cells.