

Quantitative 3D Image Analysis of a Zebrafish Xenograft Model of Glioblastoma Cell
Proliferation

by
Christine Kang

A THESIS

submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Bioengineering
(Honors Scholar)

Presented May 11, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Christine Kang for the degree of Honors Baccalaureate of Science in Bioengineering presented on May 11, 2016. Title: Quantitative 3D Image Analysis of a Zebrafish Xenograft Model of Glioblastoma Cell Proliferation.

Abstract approved: _____
Juliet A. Greenwood

Glioblastoma multiforme is the most common primary brain tumor in adults ages 45-70, with over 12,120 new cases in the U.S. predicted for 2016 alone. Glioblastoma is also one of the most difficult cancers to treat because the tumor cells tend to invade into the surrounding healthy tissue, making it difficult to completely remove all of the tumor without resulting in more damage. As a result, the 5 year survival rate remains low – less than 5% – and without treatment, median survival time is <1 year post-diagnosis. To investigate key factors in tumor cell invasion and decision-making, – including specific attachment sites, links between tumor invasiveness and distance from blood vessels, and the velocity and directionality of the tumor cells – we developed a new embryonic zebrafish xenograft model. Current work in the lab utilizes two different imaging methods – high throughput epifluorescence (via High Content Imager, HCI) and Confocal – and two different analysis packages: MetaXpress (v. 5.0.3.1) and FIJI (FIJI Is Just ImageJ, v. 2.0.0) to quantify tumor cells in the model. This paper will explain imaging and analysis protocols, compare the benefits of each analysis package based on the image source, and suggest optimal pairings and areas for improvement.

Key Words: Glioblastoma, Zebrafish, Cancer, High Content Imaging Analysis, Confocal Imaging Analysis

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Christine Kang, Author

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Background

Glioblastoma multiforme (GBM) is the most common and deadliest of malignant primary brain tumors in adults ages 45-70, with an estimated 12,120 new cases predicted for the United States in 2016.^{1,2} Despite decades of research efforts,

glioblastoma is still one of the most difficult cancers to treat because the tumor cells have the ability to invade into the surrounding tissue with finger-like projections, making it difficult to completely remove all of the tumor tissue without resulting in more

damage to the brain. (Fig. 1) But by slowing down the invasion of the tumor, a patient's survival rate can be increased. Still, the 5 year survival rate remains low -- less than 5%, and even with optimal “Stupp protocol” treatment – tumor resection followed by concurrent temozolamide and radiation therapy – median survival time is less than 1 year post-diagnosis.³

To understand the effect of basement membrane proteins, like laminin $\alpha 5$, on glioblastoma progression, the Greenwood lab developed a new embryonic zebrafish xenograft model to better understand key factors in tumor cell invasion and decision-making.⁴ These include investigating the usage of specific attachment sites, links between tumor invasiveness and distance from blood vessels, and the velocity and directionality of the tumor cells. Zebrafish are ideal model organisms for this process due to a variety of characteristics including, but not limited to their transparent bodies, presence of blood circulation and vertebrate anatomy, lack of an adaptive immune system for the first 4-6 weeks, as well as a dense microenvironment comparable to that of a human brain's.^{5,6,7} Zebrafish with transgenic modifications are also available, and the small size of embryonic zebrafish area useful for high throughput assays in a

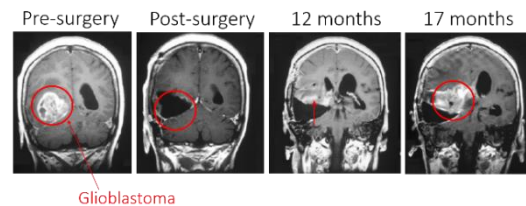


Figure 1. An MRI scan showing GBM progression. Even with optimal treatment, many patients experience tumor recurrence, because surgeons are often unable to remove all of the tumor mass without harming healthy brain cells and further damaging the brain.

96-well format. Specifically, transgenic fli fish (*Tg(Fli1:EGFP)*) were selected; these fli fish express Green Fluorescent Protein (GFP) in the endothelial layer of their blood vessels and were used to help monitor the interaction of tumor cells with blood vessels within the brain. (Fig. 2)

The Greenwood Lab's current imaging protocols involve a seven day process. Transgenic *Tg(Fli1:EGFP)* "fli" fish 0 days post fertilization (dpf) were acquired from Sinnhuber Aquatic Research Laboratory and injected with morpholinos at the 1-2 cell stage to induce knock-down of the laminin $\alpha 5$ basement membrane protein. Starting 1 dpf, the fish were continuously treated with phenylthiourea to inhibit melanogenesis and enhance

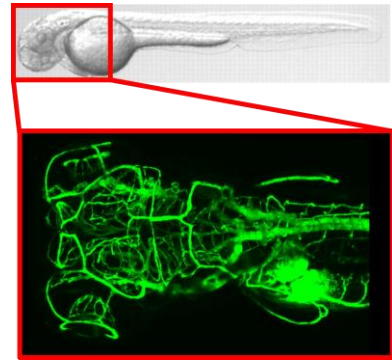


Figure 2. Transgenic zebrafish with GFP-expressing blood vessels were selected.

image quality. At 2 dpf, the fish were sedated and microinjected with fluorescently dyed cell cultured glioblastoma cells (U251 or U87). On days 3 and 7 dpf, live, multidimensional images (both GFP and red fluorescent CM-Dil) of the fish were captured using either a High Content Imager or Confocal Imager. This model will be useful for quantifying tumor cell velocity, colocalization of blood vessels and glioblastoma cells, and investigating how certain morphological characteristics affect directionality and tumor cell decision-making.

Unfortunately, while imaging technology has advanced at an incredible rate, achieving resolutions so great as to be considered "diffraction-limited" and current researchers beginning to move even beyond that to nanometer resolution,⁸ the development of quantitative analysis software has lagged behind, and most commercially available analysis toolkits still analyze images rather crudely. For example, image analysis is often limited to two dimensional planes, and even much of what is marketed as "3D" image analysis is still little more than the piecewise analysis of stacked 2D images or "z-stacks".

The Greenwood lab currently utilizes two different imaging methods – high throughput epifluorescence (via High Content Imager, HCI) and Confocal – and uses two different analysis packages: MetaXpress (v. 5.0.3.1), a commercially available software package for both capture and analysis of HCI images; and FIJI (FIJI Is Just ImageJ, v. 2.0.0), an open source analysis package. Traditionally, HCI images have been paired with MetaXpress analysis and Confocal images have been paired with FIJI analysis. This paper will explain imaging and analysis protocols, discuss the benefits and restrictions of each analysis package based on the image source, and suggest optimal pairings and areas for improvement.

Methods

Sample Preparation: Human U251 glioblastoma cells were cultured in Dulbecco's modified Eagle's medium containing 4.5 g/L glucose, 2 mM l-glutamine, and 10% fetal bovine serum following the protocols from the supplier (ATCC) at 37 °C and 5 % CO₂, and harvested at 70-90% confluency. The cells were dyed with red fluorescent CellTracker™ CM-DiI dye according to the supplier's protocols.

Embryonic zebrafish (0 dpf) were acquired from Sinnhuber Aquatic Research Laboratory and injected with morpholinos at the 1-2 cell stage to induce knock-down of the laminin $\alpha 5$ basement membrane protein. From there, embryos were continuously treated with Phenylthiourea to inhibit melanogenesis and incubated at 28 °C for two days in timed light-dark conditions (14 hours light, 10 hours dark) for optimal growth. At 2 dpf, the embryos, now hatched, were microinjected with ~ 2-4 nL of U251 glioblastoma multiforme cells near the base of the cranium. Injected (and control) embryos were moved to a second incubator kept at 33 °C, and then imaged after one recovery day using either a High Content Imager or Confocal Imager

on days 3 dpf and 7 dpf. All zebrafish handling was in accordance with Institutional Animal Care and Use Committee protocols at Oregon State University. (Fig. 3)

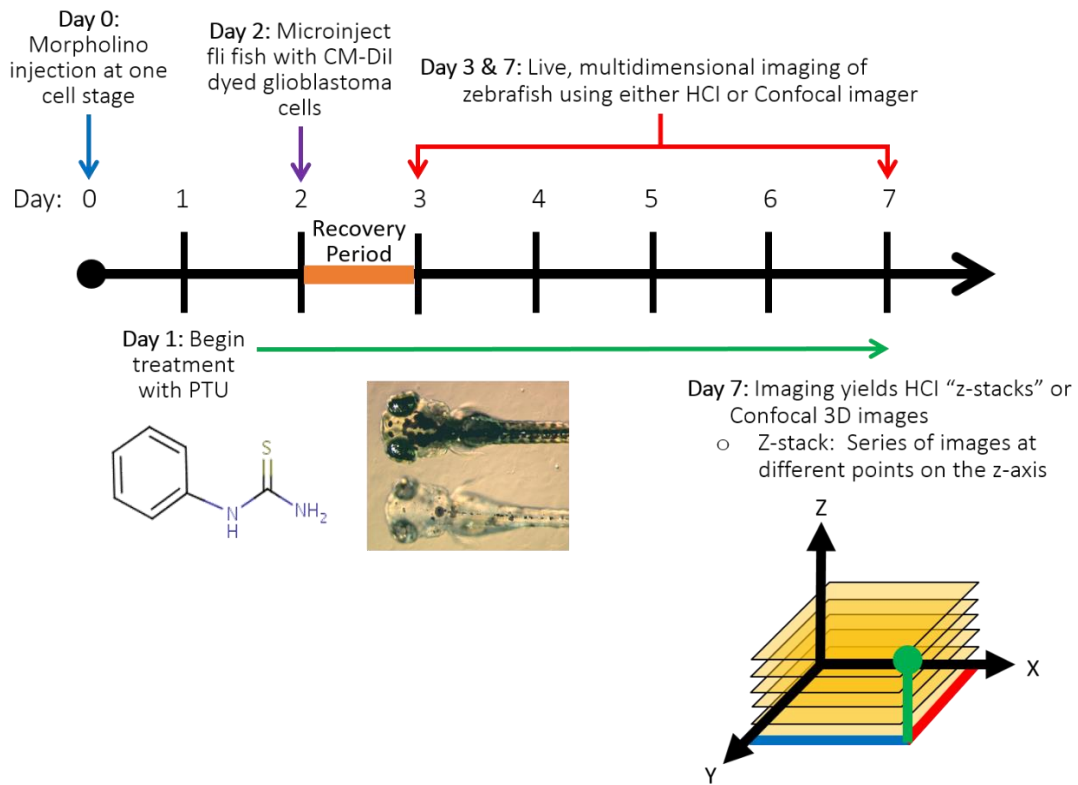


Figure 3. Overview of embryonic zebrafish xenograft model timeline.

Image Acquisition: There were two types of fluorescence imagers used: a High Content Imager (HCI, ImageXpress Micro) and a Confocal Imager (Zeiss 780 Lazer Scanning Microscope).

Output images from the HCI were in a .STK or .MDA format and contained a series of layered 2D "z-stacks" taken at pre-set intervals. Output images from the Confocal were in a .czi format and contained a series of overlapping images to build the 3D shape.

High Content Imager – No gloves at the computer.

1. Open “MetaXpress 5.1” on the Desktop.
 - a. Login information: (Subject to change, contact an administrator if these are no longer working.)
 - i. Data Source: HCI-Bridges
 - ii. Login Name: CgrbHci
 - iii. Password: SinnhuberFTNL
2. In the toolbar near the top left-hand corner of the screen, click to open “Plate Acquisition Setup” and “Plate Acquisition and Control”. (Fig. 4)
3. On the left-hand menu in the work area, click “Run a Plate” >> “Open Door – Eject Plate” to open the plate reader door. Insert the 94-well plate containing samples into the plate reader with the beveled edge facing forward.
4. Click “Close Door – Load Plate” to load the plate into the imager.
5. In the toolbar, select “Devices” >> “Stage” >> “Move Stage to Image Position”
6. In the Plate Acquisition Setup window, open the “Experiment” tab and select “Load existing settings file”, click “Load Settings...”
 - a. To open a new window, then click to open the drop-down menu and load “CK_ZF_Fli_U251”.
 - i. Important settings:
 - “Objective and Camera” >> Magnification: 10X Plan Fluor
 - “Plate” >> Plate Name: Greiner 96W half -675097
 - “Sites to Visit” >> Site acquisition mode: Single site
 - “Acquisition Loop” >> Number of Wavelengths: 3

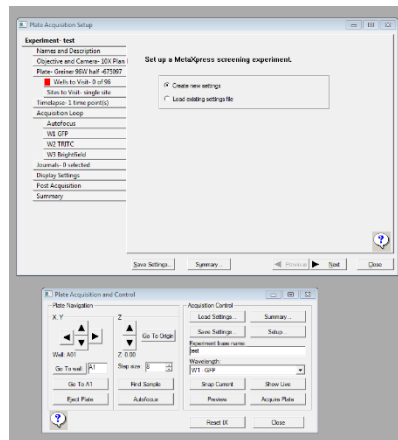


Figure 4. Screenshot of the MetaXpress program with “Plate Acquisition Setup” and “Plate Acquisition and Control” menus

- “Autofocus” >> Laser-based Focusing >> Well to well autofocus: Focus on well bottom (Fig. 5)

7. In the toolbar, select “Apps” >> open “Multidimensional Acquisition”

- “Main” tab: Select “Multiple Stage Positions”, “Multiple Wavelengths”, and “Z Series”
- “Saving” tab: There are options to change the Description, select a Directory, and change the Base Name for the newly created .MDA files.

Note that the base name will stay constant with the program adding on additional pieces to

designate each file created, i.e. if the base name is

“CK_ZF_Fli_U251_01012016”, subsequent files will be named

“CK_ZF_Fli_U251_01012016 s1”, “CK_ZF_Fli_U251_01012016 s2”, and so

on...

- “Wavelengths” >> Number of Wavelengths: 3

- “W1: GFP” (Fig. 6):

- If zebrafish have GFP vessels, exposure should be 25-50 ms
- If no GFP vessels, the Number of Wavelengths in Step 7c is 2

- “W2: Brightfield” (Fig. 7):

- Exposure: 25 ms

- “W3: TRITC” (Fig. 8):

- Exposure: 100-300 ms, depending on cell brightness intensity

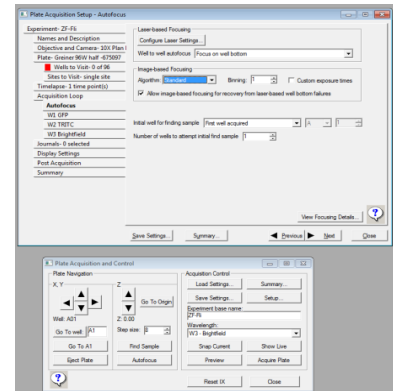


Figure 5. MetaXpress screencap to load new acquisition loop

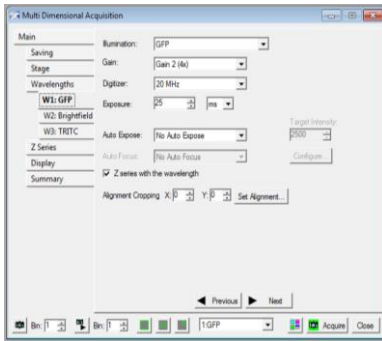


Figure 6. MetaXpress screencap for GFP settings

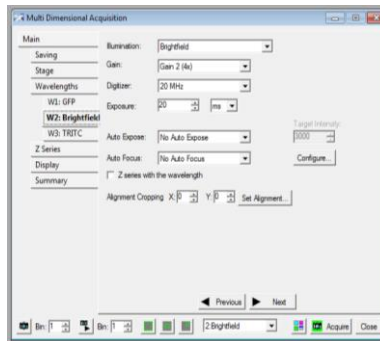


Figure 7. MetaXpress screencap for Brightfield settings

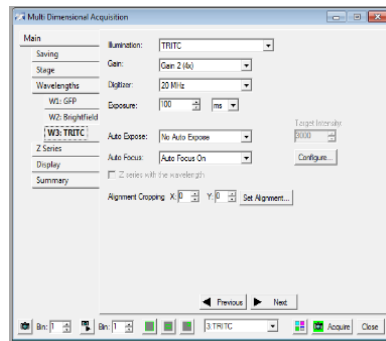


Figure 8. MetaXpress screencap for TRITC settings

d. “Z Series”

- i. Loop order: “Acquire Z series for one wavelength at a time”
- ii. Step Size: 8 μm
- iii. Number of Steps: 30

8. In the Plate Acquisition Setup window, go to the “Wells to Visit” tab

- a. Left-click and drag to highlight your wells of interest
- b. Right-click on your first well to focus the camera on that particular well

9. In the Plate Acquisition and Control window, click “Find Sample”

- a. In the drop down menu under “Wavelength”, select “Brightfield”, then click “Show Live” to see the wells in real-time
- b. Under “Z” click the up and down arrow buttons to use the real-time images to find the top of the head of one of the zebrafish
 - i. Click the center of the fish’s head to re-center the fish in the image
 - The top of head should be in focus and show some pigment
- c. Change the Wavelength to TRITC. Click the up button until you reach the edge of the bottom-most cells inside of the zebrafish brain (Should glow bright red under the filter.)

- i. Click down 15, 8 μm steps to get to the center of the injected cell mass
- ii. Click “F2: Stop” to stop the live-capture

10. Go to the Multidimensional Acquisition window and open the “Stage” tab.

- a. Change the “Position Label” to reflect the well the sample is in. This keeps track of the wells imaged and retains this information for use later on during MetaXpress analysis.

- b. Click the “+” button to add the currently focused location to the “Positions” queue.

- i. Since the naming scheme automatically names the images s1, s2, ... note the order in which the wells were imaged (Fig. 9)

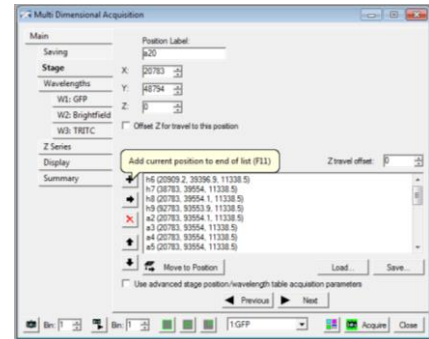


Figure 9. MetaXpress screencap for Multidimensional Acquisition

11. Start again with next well. Repeat Steps 8b – 11b until all wells have been inputted for imaging.

12. When the locations of the samples in each of the wells have been noted in the “Positions” queue in the Multidimensional Acquisition window, go to the “Summary” tab in the MDA window.

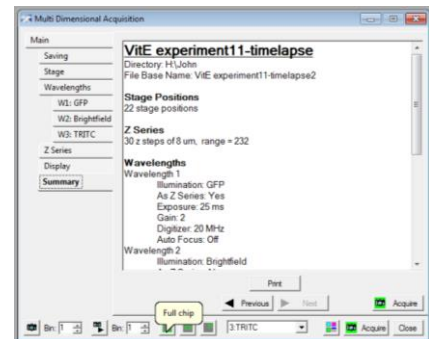


Figure 10. MetaXpress screencap for Multidimensional Analysis “Full Chip” and “Acquire”

- a. Click the “Full Chip” button so that the full image is taken instead of a single quadrant. (Fig. 10)
- b. Click “Acquire” and the program will run through and image each of the locations in each of the selected wells and save the .MDA image files in the directory specified in step 7b.

Confocal Imager

Speak with Julie Greenwood to get training for Confocal Imager use. Generally settings for

Confocal imaging are outlined in the table below:

Table 1. Zeiss LSM 780 Confocal settings for proliferation assay with zebrafish xenograft

Objective:	Plan-Apochromat 20x/0.8 M27	
Mode:	Z-stack	
Smart Setup (Detectors and Lasers):	<ul style="list-style-type: none">• TRITC• eGFP• Smart Setting	
Acquisition:	<ul style="list-style-type: none">• Bits/Pixel = 16• Frame size = 512x512• Line speed = standard• Line averaging = 1• Line Direction = ->	
Laser:	<ul style="list-style-type: none">• 561 laser<ul style="list-style-type: none">▪ Section size = 0.2um or 1.97 airys▪ power = 2 – 5▪ gain = 300 – 600	<ul style="list-style-type: none">• 488 laser<ul style="list-style-type: none">▪ section size = don't adjust▪ power = 2 – 3▪ gain = 300 – 500
Z-Stack:	<ul style="list-style-type: none">• Optimize<ul style="list-style-type: none">▪ Optimal section• Stack size – 70 – 130	

Image Analysis: There were two different image analysis packages used to count cell numbers: Molecular Device's MetaXpress High-Content Image Acquisition and Analysis Software version 5.0.3.1, and ImageJ's FIJI version 2.0.0. Generally, MetaXpress uses a series of customizable "Journals" to integrate the software's offerings into a single executable program. As a first step, the Journal used in the lab generally compresses the 3D images into a single 2D "Best Focus Image" by taking the brightest, most saturated pieces of each image in the z-stack, and layering them to form a single compressed image. Then, the Journal identifies the saturated pieces MetaXpress considers above a certain brightness threshold in this new single image, uses a "segmentation" feature to breaks down these thresholded pieces by a pre-set size, then counts these individual pieces to output a cell count to a spreadsheet. In contrast, FIJI auto-contrasts

each image in the set, stitches them together to form a single, cohesive 3D image, then analyzes them in three dimensions, counting each discrete fluorescent output within a certain size range as a single cell.

MetaXpress Analysis

For images from HCI, Confocal images can be analyzed exactly the same way, but must be first converted from its native .czi format to a .TIFF format in ImageJ – More detailed steps can be found in the black binder labeled “Zebrafish Xenograft Procedures” in the bookshelf next to the analysis computer.

1. Login information: (Subject to change, contact an administrator if these are no longer working.)
 - a. Username: moldev
 - b. Password: SinnhuberFTNL
2. Open MetaXpress software on Desktop
 - i. Log into file server - password: SinnhuberFTNL
 - b. Go to the toolbar “File” >> “Open...” to open your images from the HCI_Data folder on the Bridges1 file server (or whichever Directory they were saved to)
 - c. Open your .MDA files
 - i. Close the GFP and Brightfield images, keep the TRITC file open
3. Select from the toolbar “Process” >> open the “Stack Arithmetic” window
 - a. In the “Stack Arithmetic” window, select “Best focus” (Fig. 11)
4. From the toolbar “Log” >> “Open Data Log...”

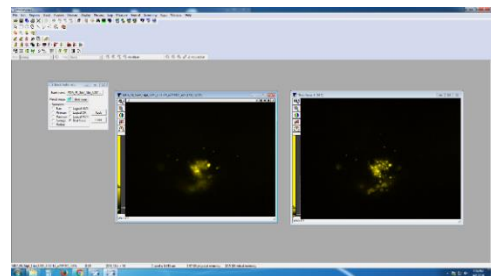


Figure 11. Screenshot with images before (left) and after (right) utilizing the “Best Focus” option in MetaXpress

- a. In the “Open Data Log” window, check “Dynamic Data Exchange [DDE]”, click OK
 - b. In the window that pops up (“Export Log Data”), select the following to export the data to a spreadsheet:
 - i. Application: Microsoft Excel
 - ii. Sheet name: DataLog
5. From the toolbar “Log” >> “Open Summary Log...”
- a. Check “Dynamic Data Exchange [DDE]”, then click OK
 - b. In the window that pops up “Export Log Data”, select the following to add a Summary sheet to the previously made spreadsheet:
 - i. Application: Microsoft Excel
 - ii. Sheet name: Summary
6. Click on the Best Focus TRITC image made previously in step 3a, then in the toolbar “Apps” >> “Multi Wavelength Cell Scoring” (Fig. 12)

- a. In the “Multi Wavelength Cell Scoring” window, enter in your desired analysis parameters. Here are some typical parameters:

- i. Segmentation
- ii. Approximate min width: 4 μm
- iii. Approximate max width: 4 μm
- iv. Intensity above local background: 100 graylevels

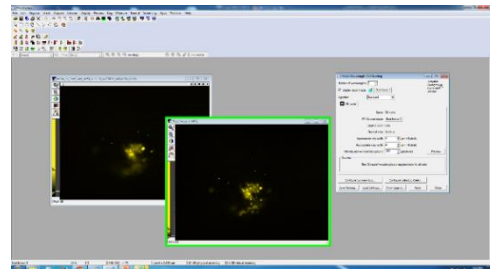


Figure 12. MetaXpress screencap for Multi Wavelength Cell Scoring settings

- b. In the drop-down menu next to W1 Source Image, select the Best Focus TRITC image that you made in step 3a
 - c. Select “Configure Summary Log” and “Configure Data Log” and make sure that “Enable All” is selected for the parameter configuration. Any unnecessary data can be deleted later.
 - d. Select “Preview” to see whether the entered scoring parameters work. There will be a white overlay on the image to show where the program has identified that there are cells. This overlay does not have to fit perfectly over the cells, however check to make sure that the software is not counting an object as a cell if there is no cell present. If the overlay fits well, select “Apply” for the data to be exported to the open Excel file created in steps 4-5 for further later processing.
7. Repeat steps 2-3, and 6 for each .MDA image. The Excel file will continue to add on the new data every time the Multi Wavelength Cell Scoring app is run.

8. FIJI

1. Open/run (Fiji is Just) ImageJ v. 2.0.0 from the Desktop
2. Open toolbar “File” >> “Open...” to select the .czi file of interest
3. Open toolbar “Image” >> “Color” >> “Split Channels” (Fig. 13)
 - a. Keep the TRITC (red fluorescence) and close the GFP channel
4. Open toolbar “Process” >> “Enhance Contrast”
 - a. Saturated pixels: 0.1%
 - b. Normalize, Process all slices, and Use stack histogram should be selected

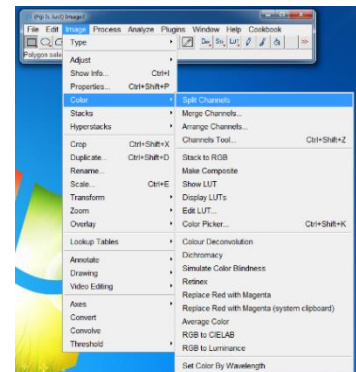


Figure 13. FIJI screencap splitting the different channels in Confocal .TIFF files

5. To filter, open toolbar “Plugins” >> “3D” >> “3D Fast Filters” (Fig. 14)

- a. Filter: Median
- b. Radius X pix and Radius Y pix should be equal and = 3 pix
- c. Algorithm: Parallelized
- d. Nb cpus: 2 (select the maximum)

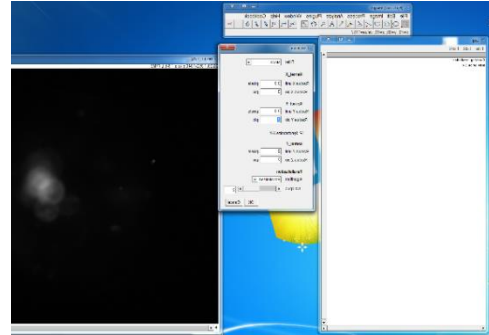


Figure 14. FIJI screencap for 3D_Filter window settings

6. Open toolbar “Image” >> “Adjust” >> “Threshold”

- a. In the drop-down menu on the left, select “Otsu” instead of “Default”
- b. Select “Dark Background” and “Stack Histogram”
- c. Click the arrow buttons in the window left

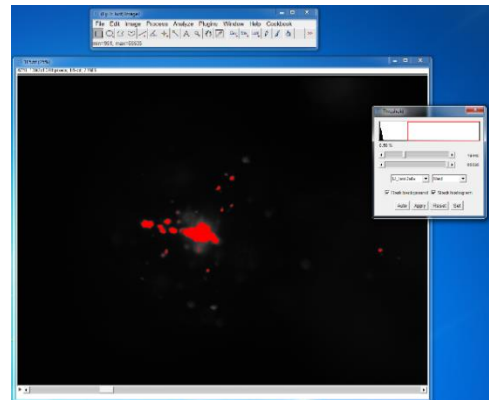


Figure 15. FIJI screencap for Thresholding. The parts of the image highlighted in red should cover at least in part all of the visible cells in the image.

and right to make sure the image is thresholded properly (make sure cells aren't double counted, but also make sure that cells are not missed either). Click Apply. (Fig. 15)

7. Open toolbar “Analyze” >> “3D Object Counter”

- a. Set Size filter min.: 20, max.: Very large
- b. Check Maps to show: Objects

Findings & Discussion

Clearly, fluorescence images taken via a Confocal are superior to those taken with a High Content Images in terms of image resolution and quality. That being said, HCI images are often

more ideal for completing preliminary tests and high throughput screening assays requiring that a larger number of samples are tested at once. The HCI's 96-well format also allows for much smaller volumes of samples to be tested at one time, making it a more cost-effective option for toxicological screening of different compounds including nanoparticles.

In terms of discussing the benefits and limitations of the two analysis software packages, images analyzed using FIJI benefit greatly from its 3D cell counting features. Despite this, the program lacks a proper segmentation feature, so it often overcorrects for potential double counting of cells and causes it to identify cell clusters as single, enormous warped cells. This is because the cell counting program is designed to identify fluorescence outputs that overlap across different planes as z-directional extensions of each other. This simple method is great for avoiding double counting of excited fluorescence that have "bled" from one plane to the next, but in situations where cell clusters must be appropriately segmented because individual cells are layered on top of one another, this method falls short and counts these discrete layered cells as a single shape. That being said, FIJI offers some customizability here and allows users to input an appropriate size range from which it can count the shapes it finds within a 3D image, however because it lacks a complementary segmentation feature, the program then completely fails to identify shapes larger than its specified range. Based on this, I would suggest FIJI as a great analysis software for fluorescence images with discrete, spaced out objects, i.e. like Zebrafish Xenograft model images with low cell numbers and high cell dispersity.

In contrast, MetaXpress allows for better cell segmentation, but requires high contrast between cells and background in order to avoid picking up background as additional cells to be counted. This particular program is also only able to count cells in 2D so images must be compressed into a single "Best Focus" image before they can be processed. It is for this reason

that Confocal images tend to suffer when processed using MetaXpress. Specifically, while Confocal images tend to have much higher resolution, they have lower contrast, so Best Focus compression tends to cause the images to lose intensity of individual cells. As a result, when cell segmentation occurs, the program must use a much lower threshold, and therefore counts imperfections in the image itself -- which do not even appear to the naked eye in the original 3D images -- as low intensity cells. As a result, images that should have ~30 cells are counted as >1200 cells. The benefits and limitations for each imaging type and analysis software is summarized in Tables 2 and 3 below:

Table 2. Summary of key benefits and limitations for High Content vs. Confocal Imaging

High Content Imaging	Confocal Imaging
<u>Benefits:</u> <ul style="list-style-type: none"> • Better for screening/high-throughput (96 well format) • Less expensive • Can be trained in-house <u>Limitations:</u> <ul style="list-style-type: none"> • Image resolution is lower • More background 	<u>Benefits:</u> <ul style="list-style-type: none"> • Higher resolution and better image quality • Less background in images <u>Limitations:</u> <ul style="list-style-type: none"> • Costly • Requires training from the CGRB

Table 3. Summary of key benefits and limitations for MetaXpress vs. FIJI

MetaXpress	FIJI (ImageJ)
<u>Benefits:</u> <ul style="list-style-type: none"> • Has (better) cell segmentation <u>Limitations:</u> <ul style="list-style-type: none"> • Requires high contrast between cells and background • 3D images must be converted to 2D “Best Focus” images for analysis <ul style="list-style-type: none"> ○ When paired with Confocal images, decreases brightness/intensity of individual cells 	<u>Benefits:</u> <ul style="list-style-type: none"> • Allows for cell counting in 3D <u>Limitations:</u> <ul style="list-style-type: none"> • Lack of proper segmentation features <ul style="list-style-type: none"> ○ Overcorrects for potential double counting of cells ○ Identifies cell clusters as single enormous cells with multiple projections

Recommendations

Overall, MetaXpress is most compatible with HCI images, and would be best for basic cell counting from images taken with the HCI. Likewise, FIJI works best with Confocal images.

Future programs or journals to improve upon existing imaging software should be able to:

- Support user inputs: As an open source software, FIJI offers considerable customizability, but requires higher levels of programming skills in order create new analysis tools for use. Conversely, MetaXpress lacks customizability options apart from Journals, limiting users' ability to create new content with which they can analyze images.
- Consider incorporating a size filter: Users will then be able to incorporate data for average cell size as well as acceptable size ranges. This may not be feasible in MetaXpress, but should be possible in FIJI.
- Count cells from "stacked" images across different vertical planes: In short, apply FIJI's 3D analysis features and allow for "double counting" to be avoided in another way by utilizing size filter information to both: 1) Avoid missing cells stacked vertically in cell clusters, and 2) Keep the program from counting cell clusters as single, extraordinarily large cells.
- Increase contrast (or work consistently despite lower thresholds): This way, the image analysis software will be able to differentiate between fluorescence from cells vs. background properly.

One general suggestion to achieve these aforementioned specifications would be to develop a program in FIJI that allows users to input average cell volumes as well as the distances (if available) between images in the z-direction. From here, the program should be able to

calculate the volume of the cell masses, then divide these masses by the input cell volume to output a cell number. This would allow for more accurate segmentation (especially in FIJI) using volume as opposed to diameter (as is in MetaXpress). Further investigation of this idea by John Gamble, the graduate student in the lab, has resulted in preliminary evidence that suggests that the addition of basic segmentation elements in cell proliferation quantification via FIJI may allow for more statistically precise data. (Fig. 16 below) This was indicated by consistent decreases in the dataset standard error of the mean and p-values when image analysis included simple volume-based segmentation versus the control. More experimental controls (including double blind studies with larger datasets) may provide more significant evidence that these simple additions to the analysis protocol will improve our analysis procedures.

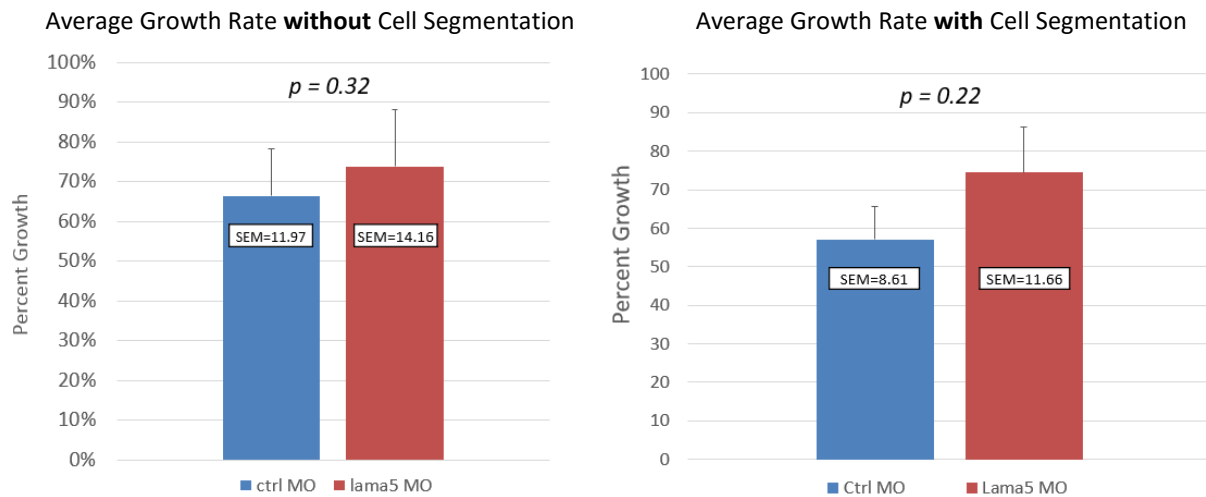


Figure 16. Some of John’s preliminary findings comparing average growth rate quantified without and with the addition of basic volume-based cell segmentation. There were 60 control fish and 39 Lama5 MO fish.

Another potential way to improve current procedures may be to make some changes to the experimental methods during sample preparation and ensure that injected glioblastoma cells are more dispersed as of day 2. This would allow for some of the software’s issues with

discriminating between and counting individual cells from cell clusters to be mitigated without changing analysis protocol.

A final possibility would be to consider changing dyes from the “dirty” red fluorescent cell membrane CM-DiI dyes currently used to nuclear stains or using glioblastoma cells with a fluorescent tag incorporated in its nucleus. This also allows for some of the issues of counting individual cells from cell clusters to be mitigated without changing analysis protocol. However, some may argue that modifying the glioblastoma cells themselves will make the xenograft model less clinically relevant.

That being said, our model is already somewhat distanced in terms of clinical relevance. Clearly, the model is in embryonic zebrafish lacking an adaptive immune response rather than in humans with the disease itself, and our cells are immortal and relatively uniform rather than primary and multiforme like the cancerous cells that are typically present in a glioblastoma patient. Despite these differences, however since this model really focuses on utilizing embryonic zebrafish and glioblastoma cells in order to investigate the cell biology-based mechanisms of proliferation and invasion throughout the brain microenvironment, even if changing this feature of the cells will decrease its clinical relevance, it should not affect its scientific relevance.

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