Distinguishing the Data from the Dark: Single Source Software or Microscopy Mix and Match?

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This article contains my personal views and was inspired by the many years of problem solving I have applied to imaging biological samples generated by microscopy. Although I come from a background in electron microscopy, I have for the last eighteen years worked exclusively in light microscopy and more recently in live cell imaging.

Here at Duke, I mentor fellow faculty, postdocs and students in finding the best ways, not only to image their experiments in the right microscope, but to anticipate the kind of data they expect to extract from their images. It is important for the student to appreciate *before* it is too late, how to best utilize parameters at the microscope that could make a big difference between success and disappointment in your take home images.

Hard data generated from images usually takes the form of *counting* (how many and where) or *measuring* objects of interest (how big, how bright, what shape, trajectory or velocity, and how much stuff). Sometimes we just want to see how the overall morphology of two phenotypes looks when you view them side by side in *comparative* terms. Results of this nature can only be documented with still images or movies. I still consider this hard data, and its importance is often overlooked when assembling a draft of a scientific paper for publication.

Many readers will have the means to either write their own software or modify commercially available modular imaging software into scripts, journals or macros to perform repetitive data analysis tasks on a batch of images, semi or fully automatically.

These brief notes are aimed at those individuals who for whatever reason depend on pre-made turnkey software with tools that can accommodate such tasks with a reasonable return on the time invested.

Over the years I have retained my favorite software tools, discarded those I think less useful and constantly lobbied vendors to be creative in generating easy to understand and robust software, supported by a good sense of user friendliness and truly progressive updates. I look for vendors that are willing to spend time with you educating users and responding to calls for troubleshooting with personal visits, phone support and/or Webex style online diagnostics. I cannot claim to represent the entire market so my comments will be to some extend flavored with personal bias, see disclaimer, above.

I very rarely use Photoshop. Instead, I prefer IrfanView, a shareware image browser that has a host of very nice simple tools for image editing. There are so many freeware image browsers out there now, but this one is small, can be carried on your USB stick and loads in the wink of an eye and is almost impossible to crash. One of its best uses is just navigating quickly around large sets of images so you can locate stuff that you want to work on, especially if you make Irfan the default browser for opening (upon

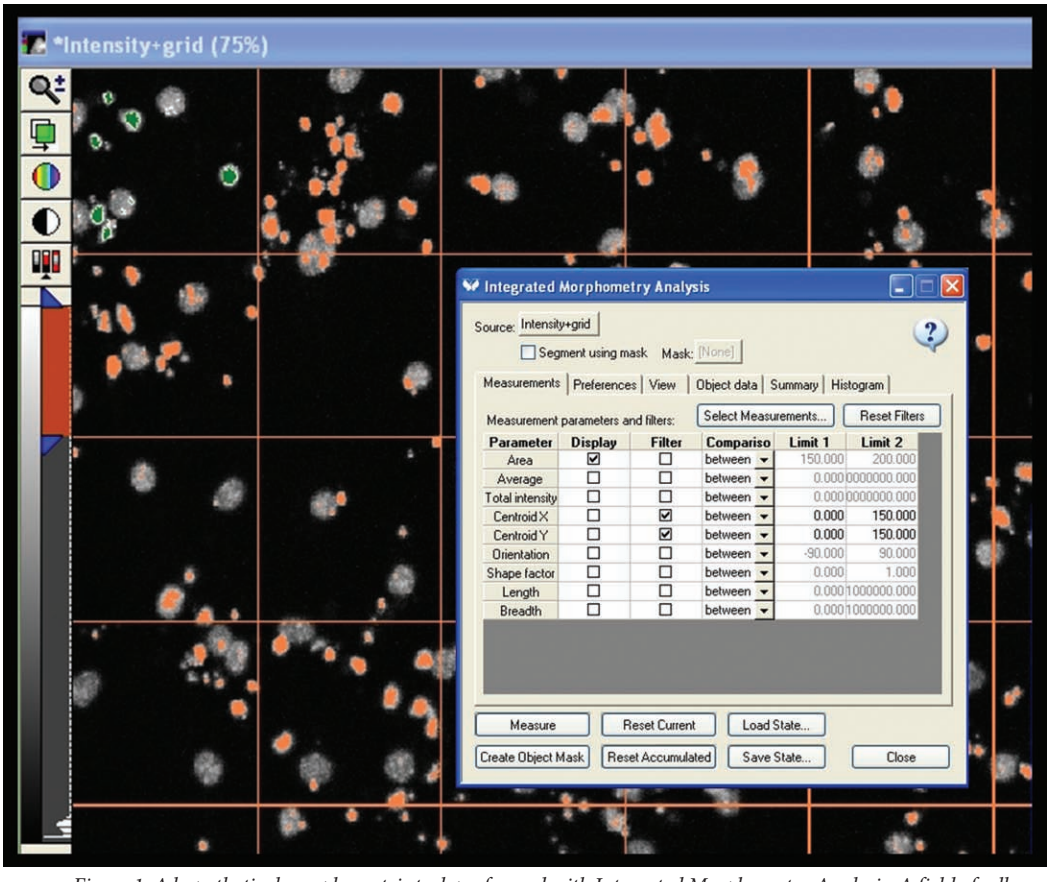

Figure 1. A hypothetical morphometric task performed with Integrated Morphometry Analysis. A field of cells rendered over a brightness range of 256 shades of grey between black (0) and white (255) also features a grid of lines in white, superimposed onto the image to divide it into 20 equal squares. The intensity threshold has been set to segment as many cells as possible as discreet, non-touching objects (orange). A preferred object size in the range150-200 pixels has been entered into the Area Display, so only cells in that size range threshold in orange. An additional centroid filter has been applied that only accepts those cells (green highlight) in the first square as scoring positive (i.e. having centroid locations in the range x(0-150), y(0-150). Note that the origin of the image (a point designated x0,y0) is the top left corner of the image. Data from tasks like this can be exported to an Excel spreadsheet.

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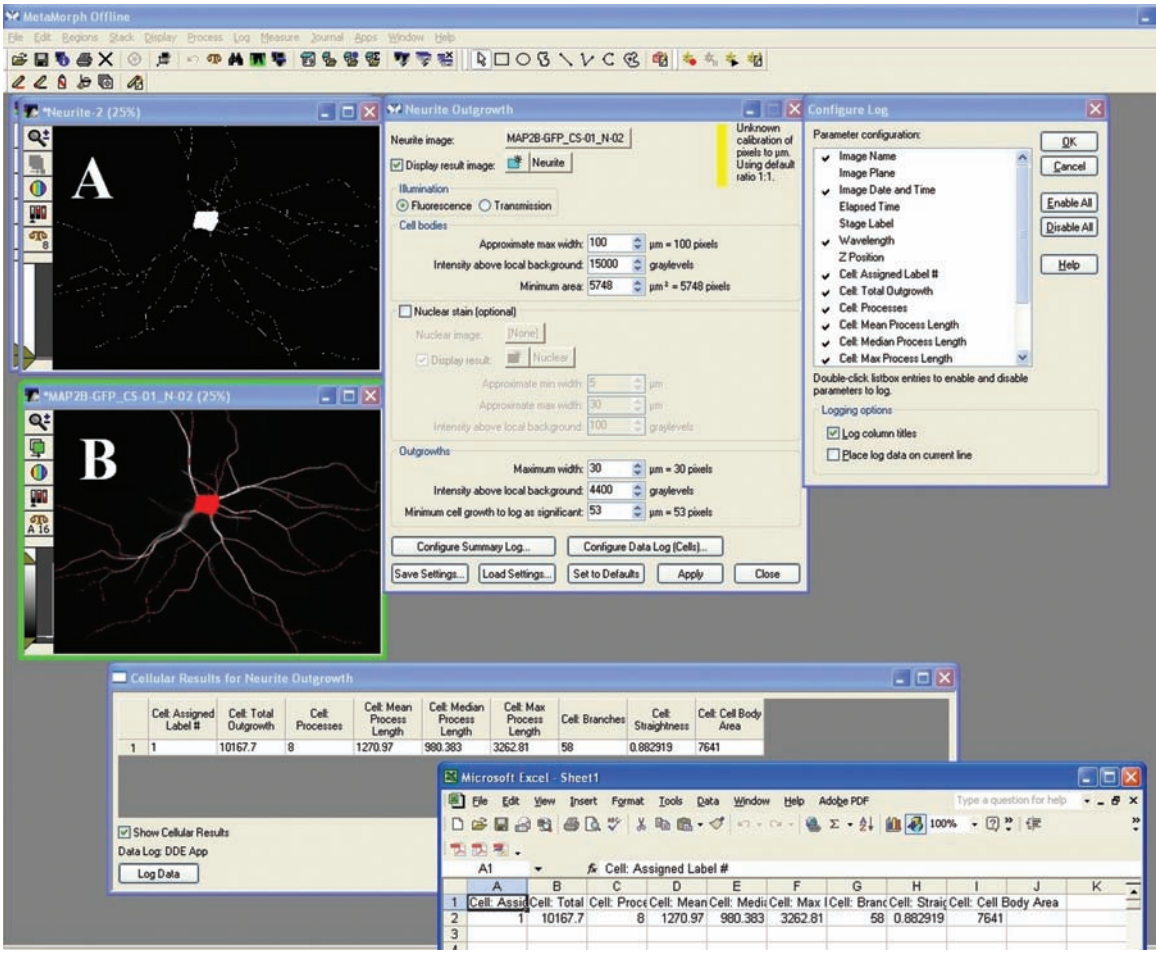

Figure 2. A neuron photographed with a sensitive monochrome camera over a dynamic range of 4096 grey levels, (micrograph B) is the starting point for Neurite Outgrowth Analysis. The user is required to enter some basic input parameters summarizing the dimensions and apparent brightness of the cell body and its outgrowths, relative to the surrounding black backround. A skeletonised cell (micrograph A) and its dimensions (Cellular Results) is output and overlaid back onto the original image (red) for visual evaluation of its fidelity. Configure Log permits the user to decide which output parameters are exported to an Excel spreadsheet (bottom).

double click) individual tiffs, jpgs and movies.

For my everyday benchtop research grade microscopes I find that snapshots acquired in 24 bit RGB color (eg H+E slides) with a Bayer masked color CCD camera, as well as false colored fluorescent images acquired with a sensitive monochrome camera are well handled with Axiovision software, which has steadily evolved over the years into a very comprehensive modular toolbox, one that can grow with your changing needs by addition of more functionality through purchase of a wide range of add-on modules. It supports some of my favorite cameras by Hamamatsu and Roper in addition to the Zeiss range of compact Axiocams.

Axiovison permits very nice control of a host of live image parameters that influence quality, prior to snapping the captured image. From that point on you can, if desired, further manipulate image quality on the post-snapped image with display mappings, brightness contrast and gamma. Display tools permit false color assignment of multiple colored fluorescent images into overlays with independent control of the component channels contributing to the final mix.

For confocal image acquisition, my Zeiss 510 software does a great job managing many different colored probes in different combinations. I especially like its ability to do a total recall of the instrumental parameters used to collect any archived image and put the instrument back in that exact same state for reproducibility. The Zeiss FRAP tools are very flexible with excellent control of bleaching precise regions of interest and more importantly it allows you to closely correlate each point on the replayed FRAP movie with its corresponding time point on the intensity recovery curve. The most recent updates into Zeiss 700/710 series have expanded on these themes with a new look to the software interface.

My Leica SP2 confocal software does a fine job too and I am very fond of the ease with which I can quickly teach novice students how to do a FRET experiment and immediately have them gain some insight into how robust the data is likely to be. The FRET wizard walks the user through just two or three pages of check boxes and variables before collecting the data, and then outputs the data into a reviewing format consistent with any web browser, so users can share FRET data easily over the internet.

My third microscope, I call the Cell Motel, is supported by Metamorph for Multidimensional Acquisition of live tissue and cell experiments. Typically, we image one or two color probes in widefield fluorescence (non-confocal), with or without brightfield, imaged in *z* stacks of multiple focus planes, at multiple tissue locations in the dish and over multiple time points. MDA is easy to teach a novice how to set up an experiment and then walk away leaving it unattended to run reliably for 4 or 5 days. It is just as easy in set-up mode or post-acquisition review mode to navigate around the different data dimensions and generate movies, so that slow changes in cellular behavior can be sped up in replay from a variety of viewpoint perspectives.

Aside from MDA, I like many other of Metamorph's software features based around its proprietary stack format which, sadly, is exportable to only one or two other software applications. Despite this, a Metamorph stack of images can easily be re-exported as a

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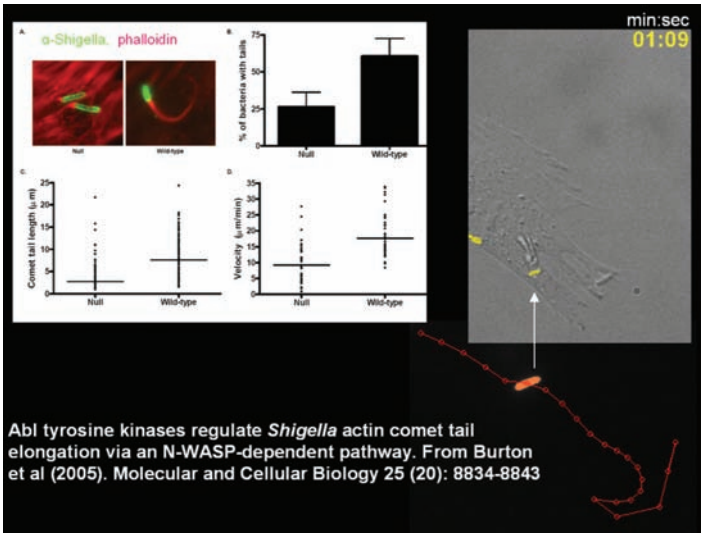

Figure 3. A scene from a timelapse movie (right) shows a single motile and fluorescent bacterium of Shigella (yellow) inside a host fibroblast co-imaged in brightfield. The same bacterium is diagramed below (orange) in the context of its complete trajectory (red track) over the entire movie. Red circles indicate the bacterium's relative positions at time intervals along the segmented trajectory, and each segment of the track represents the mean square displacement (from trigonometry) of the bacterium between consecutive timepoints. The sum of all segments gives the total distance traveled by the bacterium, used to calculate the bacterium's mean velocity. Velocity data like this often gives a valuable supplement to other related parameters of motility. In this example, fewer bacterial tails and shorter tails correlate positively with slower bacteria for N-Wasp null cells, in contrast to wild type cells that show greater numbers of tail bearing bacteria, longer tails and faster bacteria. Inset figure reproduced with permission. Copyright © 2005, American Society for Microbiology. All Rights Reserved.

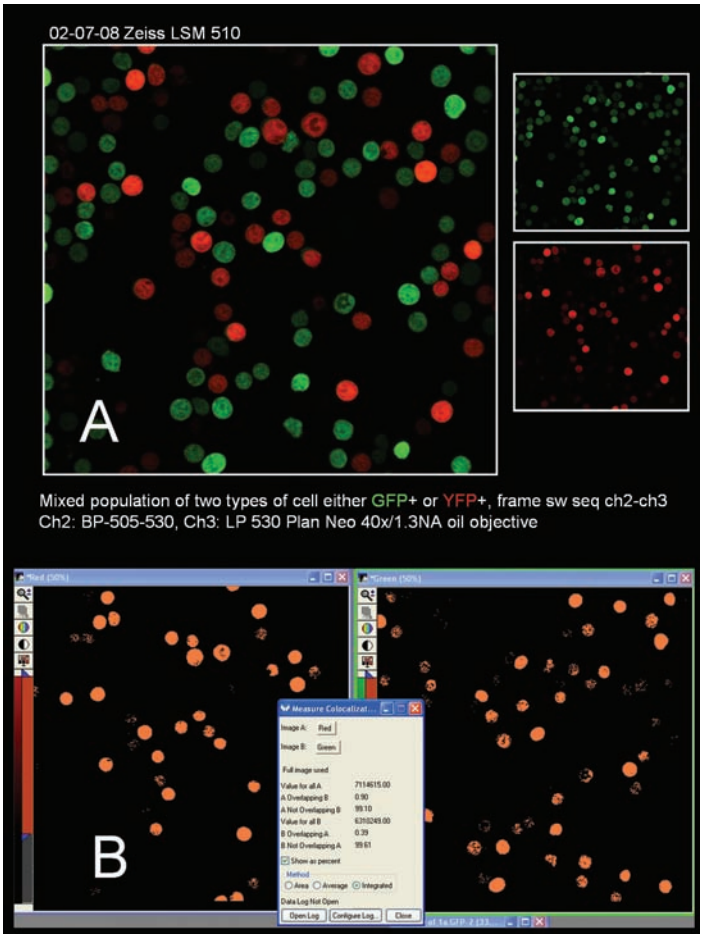

Figure 4. ***A****:Two color images were collected on a laser scanning confocal microscope with appropriate use of excitation and emission parameters to cleanly separate, with little or no signal crosstalk between, GFP enriched cells (green) and YFP enriched cells (red).* **B***: To prepare the image for colocalization measurement, the original 24 bit RGB image is separated into its green component and its red component. Next, the intensity component (monochrome, 256 greyscale) of the red and green images are displayed side by side and a suitably stringent level of intensity thresholding (orange highlight) is applied to both. The Measure Colocalization dialog displays the degree of pixel co-occupancy within the thresholded pixels and lists complementary perspectives of the data eg. all A, all B, A overlapping B, A not overlapping B, in both absolute counts of intensity and percentages. In this example, probes A (GFP) and B (YFP) are only poorly co-localized in these cells.*

series of tiffs or other popular formats for portability. The option to build task-based scripts (called journals in Metamorph) has been supplemented over the years by some very nice wizard based tools that help you make the right choices quickly. In particular the Integrated Morphometry Analysis tool allows users to identify objects by intensity "thresholding" and count, measure or filter them by a variety of complementary parameters (see example in figure1).

Another Metamorph tool that works well on suitably high contrast images is the Neurite Outgrowth analysis wizard. It needs a very well contrasted image of one or more neurites or similar branching structures radiating out from a cell body. There is a requirement that the image be collected in 4096 or more grey levels, since it employs an algorithm that exploits the high dynamic range that is typical of such tapering structures, appearing very bright at the cell body and very dim at the neurite tips (see example in figure 2).

There are also tools in Metamorph that will, in data review mode, track centroids of moving objects by thresholding or template matching, and it allows you to continuously monitor the tracking so you can intervene and correct the software if the software loses the target. This tool is a great favorite of mine (see example in figure 3) for expressing a locomoting cell's velocity (mean square displacement over time) with great confidence.

In addition, there are tools for measuring the brightness of objects in lots of different contexts and ways to express the degree of co-localization between two different colored probes on a pixel by pixel basis in any image or chosen region of an image (see example in figure 4).

One final tip, one of my favorite tools is the Print Screen button on my PC's keyboard. If you have just figured out a complex route through your myriad of choices in the software jungle, why not just snapshot the screen full of dialogs and paste the whole thing into an empty Irfan image frame? That way, next time you come back to the task you can open the screen snapped image to help you remember exactly how you left all those fiddly little checks and spin boxes. Metamorph, like many other applications, now employs "state files" to help you remember and recall the exact parameters of such tasks over and over, but the Print Screen button is universal and free. ■

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