



Wistar Institute Array Facility

Imagene.Ink

Biodiscovery Instructions for Showelab Updated 4-17-02

Before Starting...

If you have specific questions about the software call 310-306-9310, and ask for Tech Support for Imagene. They will walk you through any performance issues and help you troubleshoot the software. There are also manuals located on the shelf above the common use computer in room 304. If you have questions about settings or templates for Imagene please do not hesitate to contact us at arraysupport@wistar.upenn.edu.

The Wistar Institute Microarray/ Genomics facility is providing this information to our users to ensure that the Imagene Array analysis will be used in the same way by all users. High throughput analysis such as microarrays can yield misleading results if the data is not captured in a reliable and standardized manner and the data treatment is not consistent. We have optimized the following procedure in using facility arrays to maximize reproducibility in your analysis by reducing the background measurements and increasing the measured signal.

Capturing the Image

These instructions are for radio labeled cDNA arrays scanned on the Molecular Dynamics scanner at 50 um resolution. Please do your best to follow the facility protocols while completing your array experiment.¹ Analyze the arrays at 50um resolution on the Molecular Dynamics Storm Phosphorimager and save the file



on your local hard drive. Using the ImageQuant ImageQuant 5.1.Ink application to select each of the areas of the scan that have the arrays on it and save those selections as an independent array with a new name. Open the



file in ImageQuant tools IQTools 2.1 (2).Ink and rotate the array so that the name (i.e. MA07-03-06) is at the bottom of the array. In some arrays you will not be able to see the name written in black ink on your scan and in these cases it is important to save the arrays in the cassette to be sure that you have the correct orientation before analysis. The first time an array is analyzed the name can be the array's serial number, but second exposures must have extra information to track differences between the arrays in a database. The easiest method is to give each new exposure of the same labeling and hybridization a new number 01,02,03... and describe the changes in your notebook.

All arrays produced by the Wistar Array Facility have the following common characteristics:

- 1) The name is written at the bottom of the array.
- 2) The array element² corresponding to spot order #1 on the array template and genelist is in the top left corner of the array.
- 3) The array element corresponding to the last spot on the template and the last spot on the gene list (i.e. 9600 on the MA07) is always in the bottom right corner of the array.

There are 9600 array elements on the large format arrays (MA07,MA08,MA09,HAO4,HA05) made up of a metagrid of 384 (24x16) subgroups of 25, (5x5) spots. In this configuration the same pin printed all of the spots in a subgrid.

¹ The Wistar Array facility has a well-defined set of array protocols for use on Radiolabelled cDNA arrays such as those produced in the core facility. They can be accessed at the following Web Address <http://www.wistar.upenn.edu/genomics/links.html> .

² The term "array element" is used to denote any position on the array whether it contains cDNA or not.(e.g.. some control empty spots have been put on the array to determine background)

Examples of subgrids 1-4 on an array are shown below in figure 1, with the numbers representing the array order of the spots.

Figure 1.

1	2	3	4	5	26	27	28	29	30	51	52	53	54	55	76	77	78	79	80
6	7	8	9	10	31	32	33	34	35	56	57	58	59	60	81	82	83	84	85
11	12	13	14	15	36	37	38	39	40	61	62	63	64	65	86	87	88	89	90
16	17	18	19	20	41	42	43	44	45	66	67	68	69	70	91	92	93	94	95
21	22	23	24	25	46	47	48	49	50	71	72	73	74	75	96	97	98	99	100

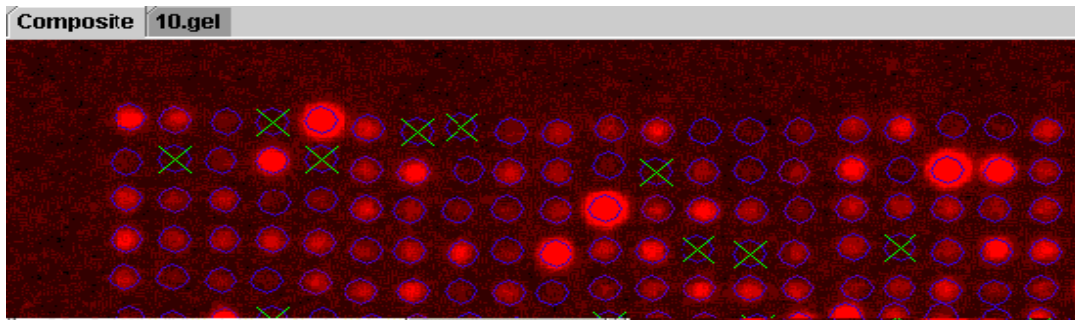


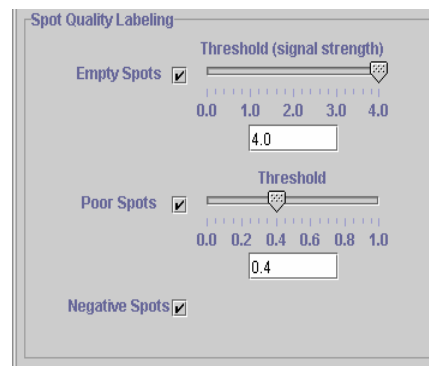
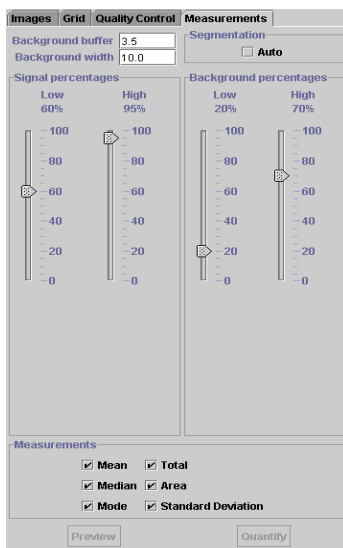
Figure 2 shows the same spots 1 through 100 on an actual HA04. Notice that the four groups of 5x5 subgrids especially in spots 1-25 cling together. This is because the first pin is slightly off center (shifted up) so all of the spots in the subgrid are also shifted up.

Instructions

Open the Imagene program.

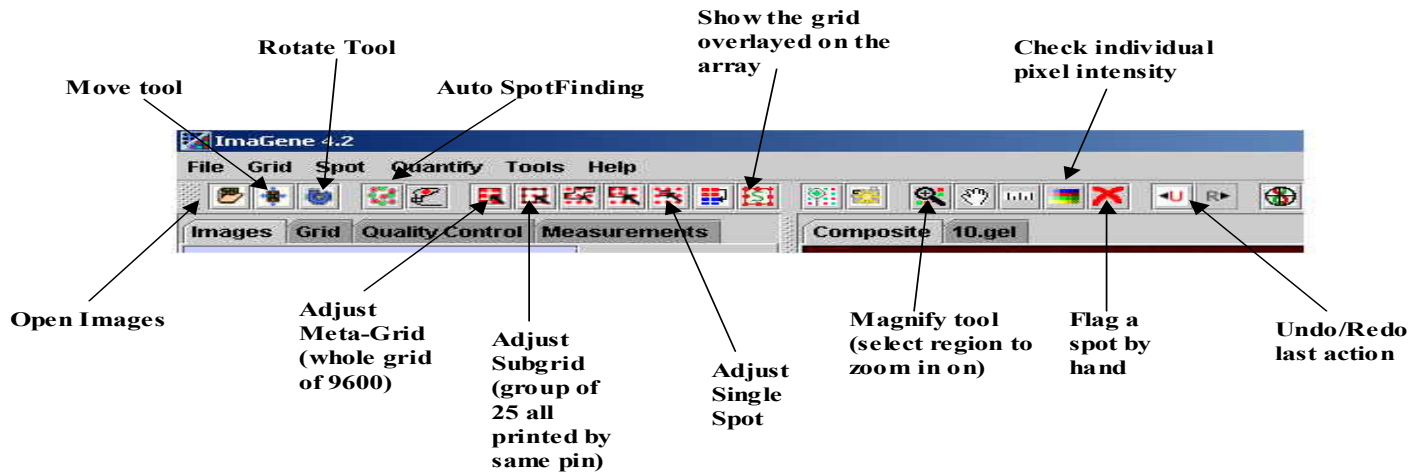
Once in the main window of Imagene you will see two main windows. The right window is where the .gel files will be viewed and the template will be overlaid and the left window is where the user controls the various tools and settings for Imagene. Remember that the program will remember the path to your image not the actual image so you should open the images from the location where you plan on saving the image. The left hand window operations are organized in four layers going left to right, starting with *Images*, *Grid*, *Quality Control*, and *Measurements*.

First click on the **Measurements Tab (below left)** and **Quality control Tab (below right)** and be sure that you have the following settings:



Closing the program or opening a new template will not affect these settings These settings will remain on your machine until you change them.

Important buttons in the Imagen Window



Click on the **Images** tab and right click in the empty *Images* window. The option “load image” will appear and left click on the words. When you do this you will be able to browse to the specific gel files that you are interested in. Adjust the image contrast with the display control at the bottom of the screen so that you can pick out the spots from background. Note that this is the first level of quality control and if you cannot adjust the contrast appropriately to see the array in this window you should consider putting the array down on a phosphorimager screen for a longer period of time.

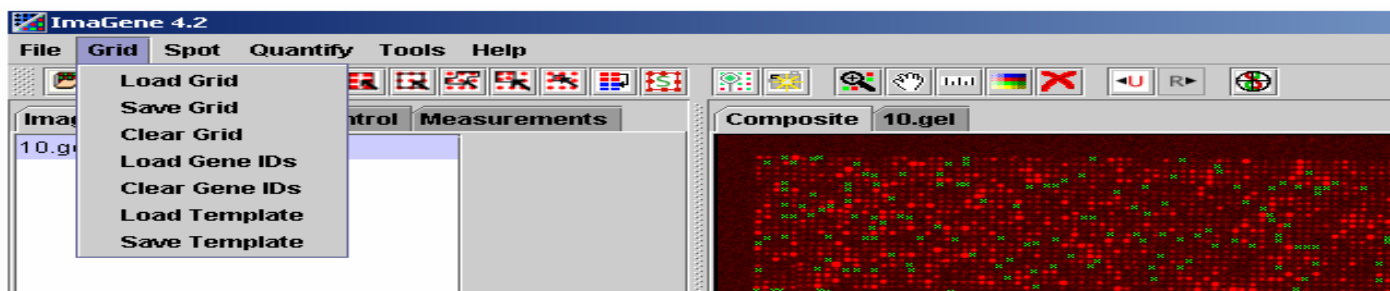
Use the **Move and Rotate** tools from the toolbar (top upper left of the screen) to orient the array so that the edges of the array are roughly parallel with the edges of the computer screen, and the writing remains at the bottom of the array. The rotate tool works by double clicking in a position on the array that you will use as the axis of rotation, then using the mouse to move the array about that axis. Hint: There are several radio buttons on the image screen such as flip horizontal and invert. Do not invert the image! If the image is inverted it will return negative numbers when analyzed. You can unclick the invert button and the array will be fine.

The Large format 9600 array element arrays (such as the MAO7 and the HAO4) are all printed using a 384-pin tool. Each pin prints one spot from each plate in what is termed a *subgrid*. For the large arrays we use 25 of the 384 well plates, which means that each of the subgrids has 25 spots in a 5x5 pattern. These subgrids are in turn grouped into a larger metagrid. There are 384 subgrids in each metagrid for large format arrays. They are grouped into 24 rows and 16 columns. If a single pin in the 384-pin tool is bent slightly then each of the 25 spots printed by that pin will all be shifted away from the theoretical center of the subgrid. Although we have tried, it is virtually impossible to bend all of the pins to correct their bending, without damaging the pins. Imagen does an excellent job of finding these off-center subgrids, if the Template is set-up appropriately. We have pre-set all of the templates for facility arrays to match the off-center pins.

Next click on the Grid tab in the menu. A drop down menu will show the tools you can use on the array.

- 1) Grids are the set-ups that help Imagen find your spot and associate the intensity value with the correct position on the array.
- 2) Gene ID's are the lists of accession numbers in the same order as the grid out put that associate the grid position to a gene identifier.
- 3) Templates are combined Genelists with Grids. We have created templates for all Wistar facility arrays. There are also Templates for many popular commercial arrays in the folder C:/Program files/Javasoft/JRE1.3/data/imagen. You will notice that this is the default setting for templates and if you hit templates you will be directed top this folder. If you are using a Wistar Array the templates

are not in this folder. Instead the Templates for all of our Mouse Human and Yeast arrays are on the Virtual O drive called “Imagene License on Fasolt”.³

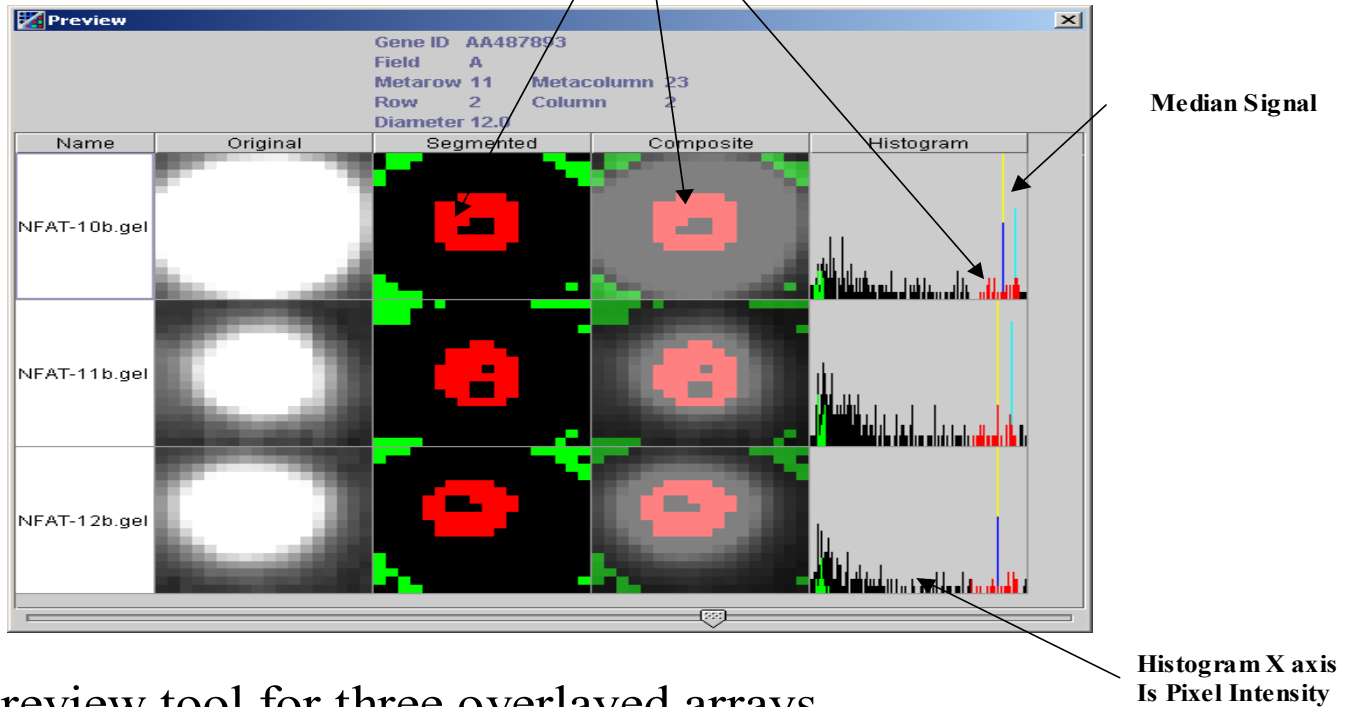


- 4) Now open the Template by going to the grid menu drop down list and hit “Load Template”. Browse to the O drive and open the folder called Imagene Templates and choose the template that matches the array you’re analyzing. Choose “place grid in saved position”. When the grid opens it will also load the corresponding Gene ID file into Imagene. All facility templates are on the O drive in “Imagene Templates”
- 5) Click on the Icon for adjust metagrid. This allows you to manipulate the entire grid. One by one click and hold onto the **corners** of the grid and move them to align with the corners of your image.
- 6) When the last corner is in place the circles should line up pretty well with all of the spots on the array⁴. Any minor adjustments can be made with the auto spot finding by clicking the icon in the toolbar at the top of the screen that looks like a wreath.
- 7) After doing the auto alignment spot finding click on the quantify tab and hit Quantify. The array will be analyzed and the appropriate quality control flags will show up on the image. Minimize the data and then toggle the zoom bar to the left until you can see the entire array at one time on your monitor. You may want to hide the grid so that you can see the flag markers on your array more clearly (consult the Important buttons graphic above). If there is an especially large group of flags in one area of the array (i.e. 25 flags in the lower right corner of the array) zoom in on this portion of the array to see whether it has been misaligned. Often this happens when the user shifts the template over too far in one of the corner and misses one of the columns or rows. Checking each corner to be sure that the array is aligned and then re-doing the auto alignment, and the quantification can fix it. This will ensure that your template is aligned.
- 8) As long as it isn’t saved the quantified data is updated in real time as you modify the alignment of the template and quality control measures. You should inspect your arrays to make sure that they are well aligned, and that the quality settings are appropriate for the arrays. One of the ways to do this is to hit the preview button in the quantify menu. Then click on any spot on the array and the segmentation of that spot will be visible (i.e. which parts of the spot are considered real signal (red), which portions are considered to be background (green), and which portions of the spot are excluded from analysis neither signal nor background (black)).

³ We have our templates here so that we can update them in one location if we need to make changes (i.e. if a clone is sequenced and found to be incorrect we will change the name on this list). We are afraid of having old copies of the templates floating around the institute so we would rather not have you copy the templates to your local hard drive. Any time that a template is modified we will send out an email to all registered users on the mailing list to update you.

⁴ The template has been set up to fit over specific arrays such as the MA07, by manually adjusting some of the Subgrids. We do this because it makes spot finding easier for users.

Red is measure of Signal intensity



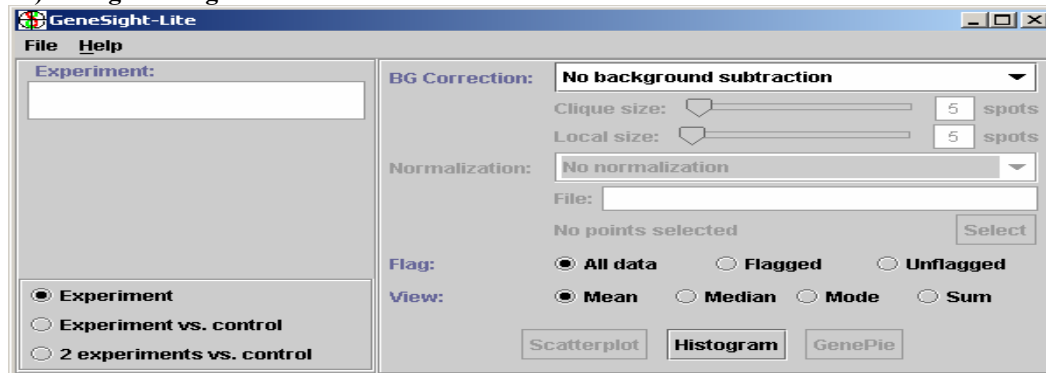
Preview tool for three overlaid arrays.

It may seem strange at first when you see that the two spots above seem to have different sizes but their red signal region is about the same size. The reason for this is that the spots themselves are all exactly the same size on the membrane, but the top spot seems larger because it has more 33P bound to it and therefore it spreads out in all directions with greater intensity. But the most intense pixels, which we are attempting to analyze here are all near the center of the spot, where the pin contacted the nylon.

Also notice that there are some pixels blacked out in the very center of the spot. We have set up the quality parameters to exclude the top 2 percent of pixels so that if one very intense spot intrudes or "bleeds" onto a neighboring weak spot, these bright pixels will be removed from the analysis of the weaker spot. This is a temporary solution, the only real solution for analyzing these very bright spots is to dilute the DNA before printing them on the array and we are doing this with upcoming printings.

- 9) After you are satisfied that the array template has aligned itself on the array image effectively, click the "Save" button at the bottom of the quantified data window. Saving the array data will also save an ".sst" file that acts as a log of the image alignment and quality control parameters. The text file can be opened in Genesight or in excel, but the sst file can only be opened in Imagene.

10) Using Genesight Lite:



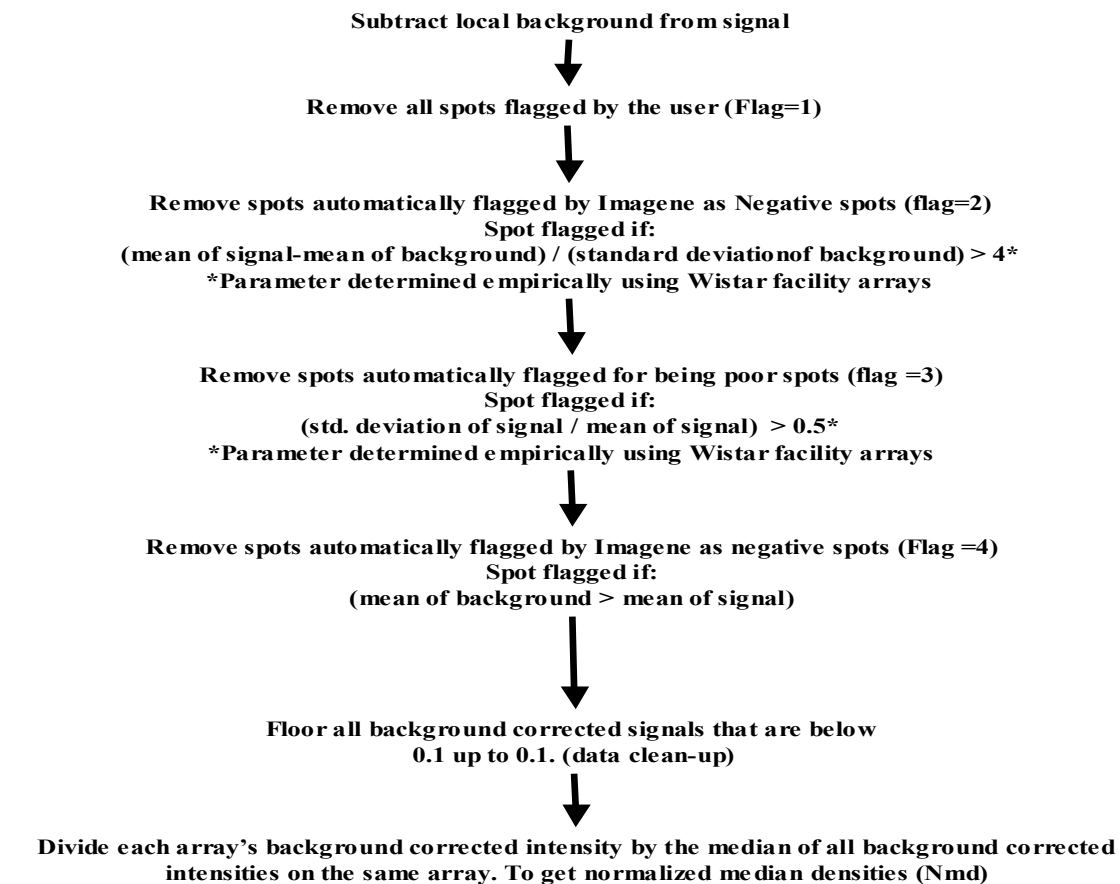
**Genesight lite cannot be used on arrays that are currently being analyzed. It can only be used on array data that has already been quantified and saved. For instance if you have two experiments and you would

like to see which genes show the most difference you can use Genesight lite to complete your analysis. **Quantify** the data and **save** the data, then click on the Genesight. Choose experiment vs. control and two separate windows will open “Experiment” and “Control”. Then right click in the experiment window select one of your data sets from the drop-down menu and then right click in the control window select another dataset from the drop down list. Select “Local background subtraction” and “Use all spots” for normalization. Check “unflagged” and “median” view. Once you select scatter plot you can view the graph of one array vs. the other. If you select a point on the plot you will also be able to view the individual spot images on each array which is helpful in determining whether the difference is real or caused by background and/or misalignment. If you select Experiment vs. Control and then use the histogram tool it will plot the ratios of all genes in the two arrays. So most of the ratios should be about 1 and the outliers will be genes that are differentially regulated between the two experiments. Genesight light also is linked to a browser which allows you to click on a spot and go to various internet databases to retrieve information about the clone.

Opening Imagene files in Genesight:

Genesight is the data analysis program that accompanies Imagene in the Biodiscovery suite. This program has several data processing tools that can be used to distinguish expression patterns. The software is also useful for normalization of array data and data clean up before analysis in any program such as Microsoft Excel, J-express or Genespring.

We use the following transformation scheme on all data:



After saving data in Imagene close the program and open Genesight 3 on the common use computer in room 304. Click the New button on the top left corner of the screen and you will be able to

import your saved Imagene data text file. The text file is by default saved in the same folder from which you selected your array image.

Open the appropriate .txt file and double-click on the file to automatically **open it in the “Experimental Conditions” window**. Alternatively you can drag and drop the .txt file into the experimental window yourself. Click **“Done”** in the upper left corner of the window, then type in your name and the date of the original labeling. The default name given to the image is the name of the gel file you opened in Imagene, but you can change the name in this window. Our practice is to use the labeling date as the date for all arrays on their first exposure. but change the name of the array if we do a second more stringent wash or a longer exposure time. This way when we enter the data into a database we will know that this was not a new labeling entirely, but rather a different exposure of the same labeling. Click **“use as default for all arrays”**, and the program will use the original image name, along with your user name and date provided on the first sample (this saves a lot of time if you are analyzing dozens of arrays).

The next window will ask you which information (columns) you want to import from the Imagene .txt file. You should **de-select mean, mode, area, total, and standard deviation**. This leaves only Median and flag as quality measures. and information about the location of the spot on the array the data will appear in the main window of Genesight, as a folder with two individual icons that look like beakers, which have the data for signal and background measurements.

Click on any one of the beakers and you will notice that the tool menu is now highlighted along the top of the screen. Select **Data preparation** from the menu. You will be asked whether or not you want to use the preview mode. Click yes. This will show you in a spreadsheet how the transformation affects your data in the first 30 genes on each of your arrays.

Go to File, pre-set data transformations. Transformation sequences, Double click on the file called **“Showelab 4-8-02 Genesight transformation.tsq”**.

The transformation will appear in the main window. This transformation modifies your data so that you can compare genes across arrays, using the scheme noted above.

Click apply data preparations, and the first thirty genes in the preview window will be modified.

Then close the window and the transformations will be applied to each of the genes on your array.

You will now see that the main data window only has a single beaker for each array corresponding to the newly normalized data. Now go to and click “generate report”. You will see the file that you are going to generate in a preview window. Save the file (default tab delimited text file) in the same folder with your .gel images. You can open this file in excel. Now that the data has been normalized you can compare the arrays across experiments.

Special Notes on Imagene Performance

Before beginning Image analysis in Imagene check to see that you have the most up to date version of the software running on your computer. You can do this by checking the Imagene virtual Drive (O) folder on the common use computer. You can check that you have the most recent version by clicking on your local hard drive and opening the directory where you installed Imagene, and opening the virtual O drive called “Imagene License on Fasolt”. Inside there is folder called “New Jar files (Updates)”. These .Jar files are the guts of the program and can be replaced quickly by copying the file and pasting it into the Imagene folder on your local hard drive. Note that we will send out an email to all registered users when we receive updates. If you have problems replacing the .jar file, email arraysupport@wistar.upenn.edu, and we can make an appointment. New versions of the Software will be made available approximately bi-monthly, and they usually are bug fixes and minor updates, but the appearance and operation of the program will not differ.

If you are having performance issues with Imagene you may not have enough RAM memory devoted to the Java platform in which all Biodiscovery programs run. You can solve this problem by re-allocating memory in the following way:

- 1) Open the directory where Imagene is installed on the computer.
- 2) Open the Imagene folder.
- 3) Open the Text file in entitled ”Imagene 4.0b Properties” in WordPad.

The file should look like this:

```
#Menu for ImaGene4.0b
#Fri May 19 17:45:19 PDT 2000
programName=ImaGene4.0b
args=-mx408m -cp .;imagene.jar;ighelp.jar com.biodiscovery.imagene.ImaGene
startDir=D:\\ImaGene
execFilename=c:\\program files\\javasoft\\jre1.3\\1.3\\bin\\javaw.exe
```

- 4) Look at the fourth line of the file. The number after the `-mx` is the current RAM allocation in kb. Reset the number to equal 80% of the RAM on your own computer. This will ensure that the program will run smoothly.⁵
- 5) Save the file.

****Do not alter any settings (i.e. Jar files or RAM allocation) on the Wistar Array facility common use facility computer. This computer will be optimized and maintained by the Facility staff.**

FAQ's

- 1) How do we re-open the Imagene file to look at the alignment after the experiment is done and the data is saved, do I have to save a log file?
 - A) Whenever quantified data is saved, Imagene creates two files, one text and one “.sst”. The .txt has all of the information about the intensity of the spots and background, in a spreadsheet format. The .sst file saves the alignment of the grid on the array and all of the quality control information such as which spots were flagged and why. If need be you can open a file months or years after the analysis was done so long as the image file is not moved.
- 2) Why do the X's and +'s stay over the template even though I imported a different array?
 - A) When you import an image into a template that has already been used during your current analysis session the template will retain the quality measures from the previous array until the new array is quantitated.
- 3) How do I get rid of the tool I am currently using in the Imagene program.
 - A) The only way to get rid of one tool is to choose another.
- 4) Why are all of the buttons at the top of the program glazed over and not responding to my clicks?
 - A) Most of the tools on the toolbar are deactivated after the program has created quantified data. During this time after quantification, but before saving, the array template cannot be moved on the array image and the array images cannot be moved. If you would like to move one or the other simply save or cancel the quantified data and the tools will again be highlighted.
- 5) If I change the quality control settings will I get different spot intensities?

⁵ Our common use computer has more than 1 Gb of RAM, so we set the `-mx` at 800. Even with this setting when analyzing dozens of 10,000 gene arrays there will be operations that require up to a minute of computing time, so be patient with large samples (or purchase a supercomputer.)

No, when you change the quality control settings you determine which spots will be flagged. Changing the settings in the measurements tab will however change the intensities of the spots. Flagging by itself does not change the data, it simply marks the data as questionable according to the standard deviation of all of the pixels in the spot and the intensity of the pixels in the spot compared to background. In our current Genesight transformation sequence all of the flagged spots will be removed from analysis and the cell where that data had resided will be deleted. If you would rather keep all of your data you can remove some of the steps in the transformation sequence. Write an email to arraysupport@wistar.upenn.edu if you have specific questions.