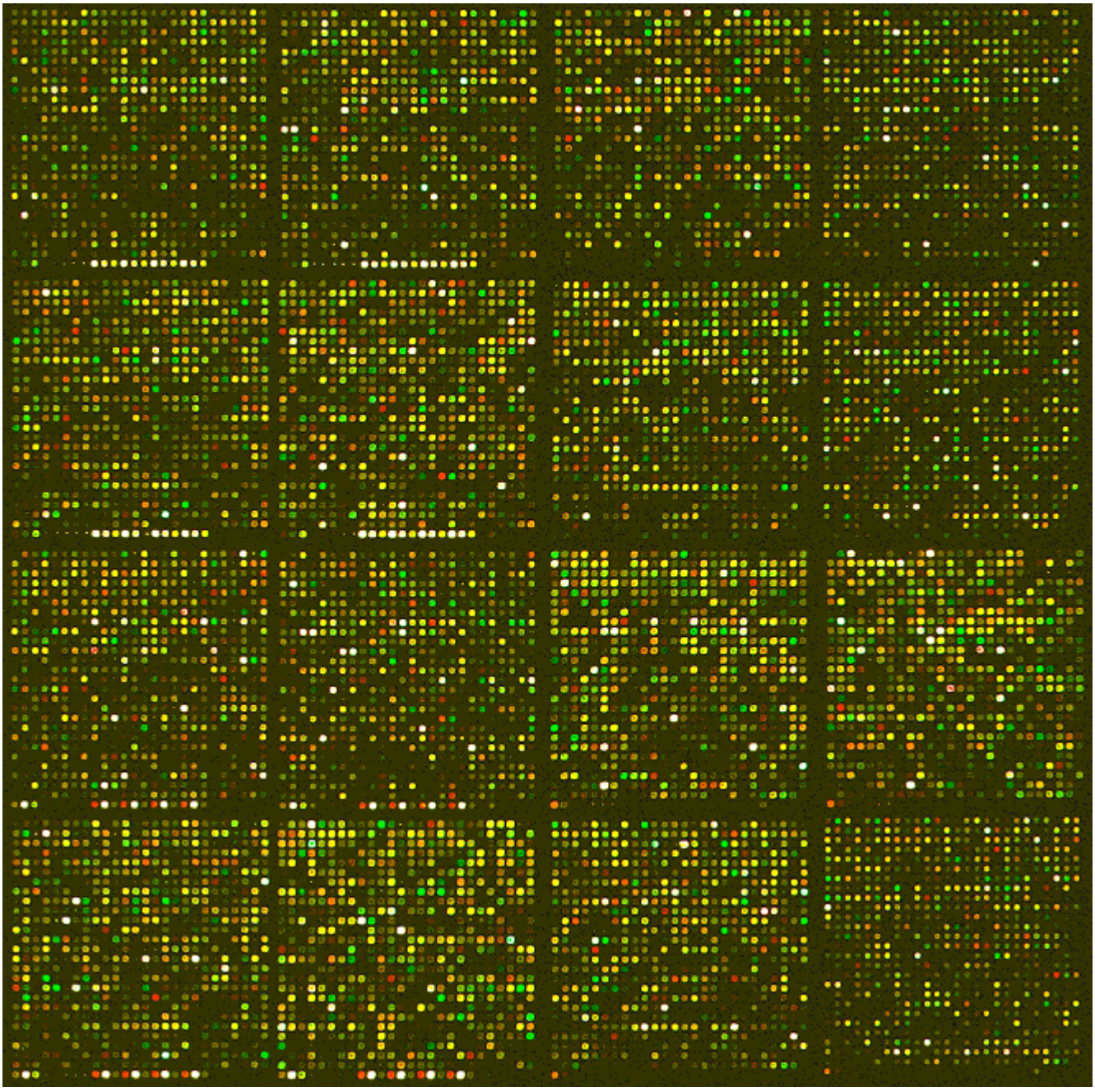
NCI Microarray Manual



Introduction

The use of microarrays for gene expression analysis has been gaining acceptance in the biological community as a significant new tool. This workshop and manual are designed as an introduction to the use of microarrays. Because of the dynamic nature of this topic, a comprehensive treatment of microarray technology is not feasible in this format. A broad range of diverse protocols exists that cover every aspect of microarray technology. We encourage you to investigate new techniques on your own. However, we will endeavor to provide you in this training class with sound methods that should work successfully in your subsequent experiments.

In general, an ***array*** is defined as an ordered arrangement of known DNA sequences on a solid substrate. A ***macroarray*** is composed of sample spots with diameters of 300 microns or larger that can be easily imaged by existing gel and blot scanners. Macroarrays are usually printed on nylon membranes and the features are spaced far enough apart to allow for radioisotopic detection. A ***microarray*** has features typically less than 200 microns in diameter with thousands of spots and is usually printed onto a coated glass microscope slide. A microarray also requires specialized high resolution scanning equipment for data collection. Array hybridization chemistries are similar to those of standard nucleic acid hybridization. While the techniques used are based on Northern and Southern blotting, thousands of gene sequences are detected simultaneously rather than only a handful at a time examined with these older methods.

Two main variants of microarrays are presently in use, the cDNA array and the oligonucleotide array. Arrayed cDNAs are typically PCR-amplified fragments from cDNA libraries or clone collections that are robotically spotted onto glass slides. Oligonucleotides may be anywhere in length from 25- to 80-mers and are either synthesized *in situ* on silica substrates or presynthesized and deposited on glass. Glass is the substrate of choice because it gives a rigid, thermostabile, optically flat surface for high-density arrays. Many coatings and surface chemistries have been developed for arraying DNA fragments, but the common feature of all of them is the presence of chemically active groups available to bind either the DNA or a linker group on a synthesized oligonucleotide.

The individual DNA molecules on the array are referred to as ***probes*** because they are of known sequence. The experimental sample of RNA that is reverse transcribed into cDNA with labeled nucleotides is considered the ***target*** as it is uncharacterized. This terminology is often confused because it is the probe that is immobilized and the target that is in the hybridization solution, the reversed of traditional blotting techniques. To avoid confusion, we will hereafter refer to the experimental sample as the target, or the “labeled cDNA”.

Oligonucleotide arrays from Affymetrix are used with a single RNA source per hybridization. On the other hand, spotted cDNA and spotted oligo arrays are hybridized with two RNA sources that are each labeled with a different fluorescent dye. The competitive hybridization between these two RNAs is analyzed by comparing the ratio of the intensity of the two fluors (such as Cy3 and Cy5). Because a ratio is used, experimental results can be compared across multiple arrays despite slight variations in the DNA concentration on the array from different print sets. Many RNA labeling protocols are currently employed for use with microarrays. The RNA can be labeled using reverse transcriptase to directly incorporate nucleotides covalently linked to fluorescent molecules. While this is the simplest method, the bulky fluors do not always incorporate efficiently during the transcription, often resulting in biased incorporation of the Cy3- over the Cy5-labeled nucleotide. In an effort to overcome this, the cDNA can be indirectly labeled by enzymatic incorporation of amino allyl-modified and/or amino hexyl-modified nucleotides into the cDNA followed by chemical coupling of the Cy3 and Cy5 fluors to the amino allyl/hexyl groups. This labeling is more efficient and less biased than direct incorporation labeling as the amino allyl/hexyl groups are smaller and less bulky than the fluorescent nucleotide molecules.

It cannot be emphasized enough that the quality of your RNA will determine the success of your microarray. While hundreds of variables exist that will affect microarray results, the most common problem continues to be the poor quality or degraded state of the RNA in the initial labeling reaction. In addition, too small a quantity of RNA in the labeling is often a contributing factor to microarray experimental failure. It is outside the scope of this workshop to teach RNA isolation techniques, but any method that gives intact RNA with a 260/280 ratio >1.8 should be sufficient. If it is not possible to get an adequate amount of RNA (>10ug), published protocols for RNA amplification and signal amplification techniques are available. We do suggest that you master the techniques presented in this workshop before you try more technically challenging methods.

Recommended Supplies for Microarray Probe and Hybridization (*March 2005*)		
SUPPLIES	**SUPPLIER**	**Catalog No.**
SuperScript™ Indirect cDNA Labeling Kit	Invitrogen	L1014-02
Dyes: Cy3 monofunctional reactive dye Cy5 monofunctional reactive dye	 Pharmacia Pharmacia	 PA23001 PA25001
Ribonuclease H	Invitrogen	18021-071
Poly (dA) 40-60	Pharmacia	27-7988-01
COT-1 DNA: Human COT-1 DNA (for human arrays) Mouse COT-1 DNA (for mouse arrays)	 Invitrogen Invitrogen	 15279011 18440016
Coverslips: 32-pin print: Lifterslips™ (25 X 40 mm) mSeries™ (25 X 40 mm) 48-pin print: Lifterslips™ (25 X 60 mm) mSeries™ (25 X 60 mm)	 Erie Scientific Erie Scientific Erie Scientific Erie Scientific	 25X40I-2-4772 25X40I-M-5227 25X60I-2-4789 25X60I-M-5439
Staining Dish/rack (10 slide)	Fisher	08-812
Slide Box (100 slide)	Thomas Scientific	6708-G28
Slide Box (25 slide)	Thomas Scientific	6708-G08
Hybridization chambers: Dual Hyb Chamber Single Hyb Chamber Single Hyb Chamber Deeper hyb chamber to accommodate thicker mSeries™ cover slip: Single Hyb Chamber	 Genomic Solutions Telechem Int., Inc. Corning Telechem Int., Inc.	 JHYB200004 AHC 2551 AHCXD
Hyb Oven	Fisher Scientific	13-247-10
Forceps	Fisher Scientific	10-295
Mini-Elute PCR Purification Kit	Qiagen	28004
High Quality Pre-filtered BSA	Invitrogen	15561-020
Centrifuge with microplate carrier assembly		

Target Preparation/Hybridization Using Total RNA

I. cDNA Generation:

Prepare separate cDNA labeling reaction for each fluorescent dye you wish to use.
A "master mix" (step 4) can be made, and the reaction increased up to 5X if needed.

1. Make dilution of 5.0 – 20.0 µg of total RNA in 16.0µl of DEPC water.
2. Add 2.0µl of 2.5µg/µl anchored oligo $d(T)_{20}$ primer.
3. Incubate at 70°C for 5 minutes. Cool on ice for at least 1 min.
4. Combine the following components for each sample in a sterile, RNase/Dnase-free microcentrifuge tube:
    a. 6.0µl of 5X First-Strand buffer
    b. 1.5µl of 0.1 M DTT
    c. 1.5µl of 10mM dNTP mix
    d. 1.0µl of RNaseOUT™ (40 U/µl)
5. Add the mixture to the annealed primer and RNA.
6. Add 2µl of 400 U/µl SuperScript™ III RT and incubate at 48°C for 2hrs.
7. Incubate at 70°C for 5 minutes to stop reaction.
8. Cool down by spinning in a microcentrifuge at maximum speed for 1 minute.
9. Add 2µl of 2 U/µl RNase H and incubate at 37°C for 20 min.
10. Add 0.5 µl of 0.5M, pH 8.0 EDTA, mix well and proceed with purification.

II. cDNA purification: (QIAGEN MINElute purification kit)

1. Add 100 µl of Binding buffer ***PB*** to RT reactions and mix well.
    Note: Can add a maximum of 2 reactions per column.
2. Apply to separate spin columns. Incubate for 1 minute.
3. Spin for 1 min at full speed.
4. Discard flow-through.
5. Add 500 µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
6. Spin for 1 min at full speed.
7. Discard flow-through.
8. Repeat wash step.
9. Discard flow-through.
10. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
11. Place columns in a fresh 1.5ml microcentrifuge tubes.
12. Add 10 µl of Elution buffer directly to the membrane. (20 µl of Elution buffer if 2 rxns were put on the column).
13. Incubate for 1 min.
14. Spin for 1 min at full speed.
15. Apply flow-through back on membrane.
16. Incubate for 1 min.
17. Spin for 1 min at full speed.
18. Discard columns, spec on Nanodrop to determine cDNA concentration.
19. Dry down in SpeedVac for 15 min at medium temp. DO NOT OVERDRY!

II. Alternative procedure – cDNA precipitation:

1. Add 3µl of 3M sodium acetate, pH 4.5.
2. Add 1µl of 20mg/ml glycogen.
3. Add 100µl of ice-cold 95% EtOH.
4. Incubate at –20°C for at least 30 minutes. The reaction can be stored at this point for several days or up to 2 weeks.
5. Spin the reaction at 13-14,000Xg for 20 minutes at 4°C. Carefully decant supernatant.
6. Wash with 0.5 ml ice cold 70% EtOH and spin at 13-14,000Xg for 15 minutes at 4°C. Carefully decant supernatant and let to air dry. A vacuum dryer can be used but DO NOT OVERDRY!

III. NHS-ester containing dyes coupling reaction:

1. Resuspend cDNA pellet in 5µl of 2x coupling buffer. (If pellet was over dried gently heat at 37° C for 15 minutes to aid in the resuspension process.)
2. The first time a tube of dye is used, resuspend in 45µl DMSO. Use DMSO provided with the kit.
3. Add 5µl of the resuspended monofunctional reactive dye to cDNA.
4. Mix thoroughly by gently pipetting up and down.
5. Incubate for 30 minutes up to 1 hour at room temp in the dark, flicking the tubes occasionally.

*IV. **Dye-Coupled cDNA Purification:** (using QIAGEN MINElute purification kit)*

1. Add 10µl of 3M Sodium Acetate, pH 5.2 to each RT reaction, mix well.
2. Add 100 µl of Binding buffer ***PB*** to RT reactions and mix well.
3. Apply each RT reaction to separate spin columns.
4. Incubate for 1 min.
5. Spin for 1 min at full speed.
6. Discard flow-through.
7. Add 500 µl of Wash buffer ***PE*** per reaction (Be sure that ethanol was added to ***PE*** buffer).
8. Spin for 1 min at full speed.
9. Discard flow-through.
10. Repeat wash step.
11. Discard flow-through.
12. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
13. Place columns in a fresh 1.5ml microcentrifuge tubes.
14. Add 10 µl of Elution buffer directly to the membrane. (20 µl of Elution buffer for 48-pin print).
15. Incubate for 1 min.
16. Spin for 1 min at full speed.
17. Apply flow-through back on membrane.
18. Incubate for 1 min.
19. Spin for 1 min at full speed.
20. Can spec using Nanodrop to determine labeling efficiency and cDNA concentrations.

*V. **Pre-hybridization:** (should start approximately 2 hours before setting up hybridization)*

Pre-hybridization buffer = 5X SSC, 0.1% SDS and 1% BSA. (Can make 10% BSA stock and filter before use or purchase pre-filtered BSA; store pre-hyb buffer at -20° C and thaw only once, warm to 42° C prior to use.)

1. Apply 40 µl of pre-hybridization buffer to the array and incubate for 42° C for at least 30 mins and up to 1 hour. (If a 48-pin print, then apply 80 µl of pre-hyb buffer to the array.)
2. Wash off the pre-hybridization solution by rapidly plunging the slide in distilled water for 2 mins, then transfer slide to 100% isopropanol for 2 mins.
3. Allow slide to air dry completely prior to use. (Can spin dry if in a rush.) (NOTE: Do not exceed 1 hour after pre-hybridization/drying before setting up hybridization.

*VI. **Setting up hybridization:***

1. Combine Cy3 and Cy5 labeled targets together (~9 µl recovered for each).
2. Add 1µl COT-1 DNA (8-10 µg/µl) and 1µl poly A (8-10 µg/µl).
3. Denature target at 100^{o}C for 1 minute, then snap cool on ice. (Final volume should be about 20µl.)
4. Make fresh 2X Formamide hybridization buffer (50% formamide, 10x SSC, 0.2% SDS) and warm to 42^{o}C just before adding to samples.
5. Add 20µl of 3X SSC to wells in hyb chamber to maintain humidity.
6. Add 20µl of 2X F-hyb buffer to samples. (Add 40 µl of 2X F-hyb buffer to samples for 48-pin print.)
7. Load 40µl sample onto microarray. (Load 80 µl onto 48-pin print.)
8. Incubate overnight (12-16 hours) at 42° C in water bath or hybridization oven.

Wash:

a. **cDNA slides:**	b. **Oligo slides:**
• 2x SSC, 0.1% SDS for 2 minutes, with occasional plunging • 1x SSC, for 2 minutes, occasional plunging • 0.2x SSC, for 2 minutes, occasional plunging • 0.05X SSC, for 1 minute • Spin 3 minutes / 650 rpm to dry	• 2x SSC, 0.1% SDS for 2 minutes, with occasional plunging • 1x SSC, for 2 minutes, occasional plunging • 0.2x SSC, for 2 minutes, occasional plunging • NOTE: The last wash of 0.05X SSC is omitted • Spin 3 minutes / 650 rpm to dry

WASHES:	2XSSC+0.1%SDS	1XSSC	0.2XSSC	0.05XSSC
dH_20:	179 ml	190 ml	198 ml	200 ml
20XSSC:	20 ml	10 ml	2 ml	0.5 ml
20%SDS:	1 ml	-	-	-

Scanning Arrays

Powering Up
The power switch is located on the left side of the scanner towards the back. In newer scanners, the power switch is located on the power supply connected to the scanner. Turn the scanner on (it can be used immediately without warm-up) and use the mouse to double-click on GenePix Pro icon. This opens the control software and initializes the scanner. (NOTE: GenePix Pro *demo* does not allow one to save data; the scanner will not operate in the *demo* application.) If an error message appears indicating that the scanner cannot be found the computer was probably started up with the scanner turned off, so you can restart the computer with the scanner on. Once the computer is on, the scanner can be turned on and off without the need for restarting the computer.

Inserting the Microarray
Once the scanner is on and initialized in GenePix Pro, the array can be inserted. Open the slide port at the front of the scanner by gently sliding the door to the left. Open the slide chamber by gently depressing the thumb toggle and lifting up. Insert the slide array side down with the NCI array number/label facing front. Be sure that the slide is resting on all four edges of the slide track. Lower the chamber top and gently roll the thumb toggle forward then back – locking the chamber down securely. Close the slide port door (you should hear the scanner hum as it draws the chamber in).

Scanning
The first step is the performance of a preview scan at low resolution to determine the exact location of the array area. Click on the hardware settings button in the bottom right hand panel on the screen to bring up the PMT settings window. Within this window, 635 nm refers to the Cy5 channel (red) and 532 nm refers to the Cy3 channel (green). Set the voltages at about 700 for each channel (the maximum setting is 1000 and is usually not necessary) and set the *lines to average* field at 1. Another option is to average more than one line, and people will do this to decrease the electronic noise during high resolution scan. Next, click on the preview scan button (top right). The large black rectangular field in the center of the screen represents the slide area. As the machine scans in both channels simultaneously, the combined fluorescence image will resolve in the slide area. Be sure to utilize the brightness and contrast slide controls at the left center of the screen. These levels can be adjusted up and down using the mouse and only affect the slide image as you see it on the screen – not the actual image itself. Typically, the hybridized area is easily observed. Once the scan has passed the hybridized area, click on the red stop button (top right panel). Next, use the view scan area tool (left center) to click-and-drag a box around the array area that you want to scan. This box can then be moved and adjusted using the mouse. If necessary, select the zoom mode magnifying tool (at left center) to magnify the image and then adjust the box as necessary. Zoom back out when finished by clicking on the undo zoom button (top left).

After designating the area to be scanned, begin a 10 micron, high resolution scan by clicking on the high resolution scan button (top right). The ratio circle at the top left of the screen will be automatically selected to allow you to see the combined fluorescence image (e.g. red and green together). By selecting the wavelength 635 and wavelength 532 circles, you can toggle between the two channels to view the fluorescence in each channel separately. You should also adjust the brightness and contrast settings to obtain optimal visualization of the signals. Remember, the scanning process is dynamic and user-interactive. The PMT settings, magnification tools, contrast/brightness settings, and ratio/635/532 circles should be adjusted on the fly to rapidly determine each PMT setting for maximal signal intensities and optimal normalization. In general, this is accomplished by scanning 1/3 - 1/2 of the array, adjusting the PMTs in the desired direction, and rescanning as necessary. Ideally, adjustment of the PMTs up or down in each channel achieves maximum signal intensities with

minimal saturation (saturated spots are colored white) and also balanced (normalized) intensities between the two channels. In general practice, spot saturation should be limited to ~1% of spots and with channels normalized the majority of spots should appear approximately yellow (as viewed with the ratio circle selected). You can also use the histogram to balance the two channels, and attempt to make them overlap on the histogram chart.

Once the scanning is completed, the images must be saved. Click on the save images button at the right center. In the *Save Images* window: 1) name your scan in the *File name* field, 2) set the *Save as type* field at *Multi-image TIFF files*, 3) leave the *Naming* field blank (i.e. no boxes checked), and 4) check both *Wavelength 635 nm* and *Wavelength 532 nm* boxes in the *Images* field. The Cy-5 and Cy-3 TIFF images will be saved simultaneously in a single file to the C drive: Axon folder: Data folder unless otherwise specified. Currently, writeable CDs, and 100 and 250 MB Zip disks (all IBM-formatted only) can be used on the ATC and 10/4B54 PCs and are recommended.

It is very important to save your files to disk when you finish with the scanner. The C and L drives "fill up" frequently and therefore must be purged of all files by erasing the contents of the Data folder! So, make sure to save your image files to disk before you leave the scanner. Once you have saved your files, please drag any folders you created to the recycle bin to help free up storage space. We periodically purge the computers at the ATC of any files that are more than three months old.

Array Analysis

GenePix Pro 5.0 Microarray Analysis software was developed by Axon Instruments and has been tailored to meet certain NCI needs. A detailed description and tutorial of the array analysis process can be found in the GenePix Pro 5.0 User's Manual which you can download from the following website as a PDF file (open with Adobe Acrobat 3.0 or higher):

http://nciarray.nci.nih.gov

The NCI Microarray Facility provides access to this software at two locations: ATC/128 and 10/4B54. As of this time, GenePix can be run on PCs only. You can purchase the GenePix software for your own lab if you desire. This will facilitate analysis of your data and reduce the high traffic flow on the facility computers designated for this purpose.

We recommend collecting your data on writeable CDs as this is the safest storage media. We discourage the use of Jazz disks (IBM-formatted only) as these are drives are not in every computer at the both ATC/128 and 10/4B54. For a few scans at a time data collection by Zip disk is also a viable option.

Overview

The goal of this analysis is to measure the intensity of Cy3 and Cy5 signals recorded in the scanned image(s) so that signal ratios (i.e. expression ratios) can be calculated for each feature on the array. This is accomplished in the following stepwise fashion: 1) image(s) are opened in GenePix; 2) the appropriate GAL file (Gene Array List) is uploaded; 3) grid blocks are aligned on the feature blocks; 4) feature indicators are fitted to the array spots; 5) measurements are performed; and 6) the Results file (a .gpr file) is saved together with a color JPEG image (a .jpg file) to your disk for deposition into the NCI Microarray Database (http://nciarray.nci.nih.gov).

GenePix Start-Up

Once GenePix Pro is open, click on the **Open/Save button** and select *Open Images...* (hot key Ctrl+O). This opens the "Open Images" window. Browse the computer for your disk, select your

image(s), and then press the **Open button**. (NOTE: If your images were saved as *single-image tiff files*, you will use the mouse to click on (i.e. select) both files while holding down the shift key. Alternatively, if the images were saved as a *multi-image tiff file*, you simply select the file with the mouse.) A color image of your array will appear. Use the magnification tool (hot key z) to zoom in on your array image. Click on the **Open/Save button** again and select *Load Array List...* (hot key Alt+Y). This opens the "Load Array List" window. Browse for your disk, select the appropriate *.gal file* (this stands for Gene Array List and can be downloaded at http://nciarray.nci.nih.gov), and press the **Open button**. You will be given two options: 1) "replace existing blocks and apply gene names", and 2) "apply gene names to existing blocks". Typically, you will select the first one (unless you have saved a grid template from a previous analysis; this is discussed below). The grid blocks will appear on top of the array image.

Gridding (block mode)

The goal here is to align the grid blocks with the array spots such that each feature indicator (these are the circles that make up the grid blocks) is relatively close to its corresponding spot. Then the *align blocks* function will automatically fit each feature indicator to its corresponding spot. There are two grid modes you need to be familiar with - one is called "block mode" the other "feature mode". In block mode, you use the mouse to manipulate the grid blocks. In feature mode, you use the mouse to manipulate feature indicators. You can select block mode by typing the hot key B, or right-clicking on the mouse for the "right-click menu" from which you can select "block mode". Similarly, you can select feature mode by typing the hot key F, or right-clicking on the mouse for the "right-click menu" from which you can select "feature mode". (NOTE: in block mode, the pointer tail has a box on it; in feature mode, the pointer tail has a circle on it.) In block mode, select the top left grid block and drag it over the block of spots. Zoom in on the block. To align the feature indicators with the spots, click on the corner and side boxes (i.e. small white boxes on the perimeter of the grid block) and while holding down the mouse button, drag the mouse to resize the grid block. (NOTE: you will get a good feel for this with practice.) When you click on a corner, the opposite corner is anchored. However, if you click on a corner and hold down the Ctrl key, all three of the other corners are anchored allowing you to flex the grid to your specifications. The grid blocks can also be moved using the keyboard. Using the arrow keys the blocks can be moved in tiny increments. Using the "+" and "-" keys on the number pad the blocks can be tilted. Remember, you do not need to align each feature indicator perfectly over each spot; just get them close and the auto-align function you will use later will fit them perfectly. Once the first block is aligned, hit the ">" key to rapidly move to the next block for aligning. (NOTE: if you plan on analyzing other arrays, you might want to save this grid template so that you don't have to align grids from scratch each time. To do this, use the "Save Settings" and "Open Settings" selections under the Open/Save button.) Once all grid blocks are aligned, press the “Align Blocks” button and select "Align Features in All Blocks". Or use the hot key Shift+F5. The feature indicators will then automatically align to each spot, resizing and repositioning as needed and flagging spots with very low signals as "Not Found". The flagging feature is very useful in that it allows us (once our data is in the database) to filter out flagged spots thus reducing the noise (i.e. false positives) in the data. (NOTE: by pressing the **options button** and selecting the analysis tab, you can define the size limits of the spots that will be measured; if the spot falls below or above the thresholds you set, it will automatically flag it "not found". You can adjust this if necessary depending on the degree of autoflagging that occurs.) After the feature indicators are aligned, switch over to feature mode (by pressing the hot key F) and move from block to block double-checking the auto-align results.

Gridding (feature mode)

In feature mode, feature indicators can be nudged around and resized for a modified fit if you desire, and flags can be removed and added as you deem appropriate. To reposition a feature indicator, select the feature indicator by clicking on it and use the arrow keys on the keyboard to move it. To resize a

feature indicator, hold down the Ctrl key and use the same arrow keys. To remove or add flags, select the feature indicator and right click to pull up the "right-click menu". Select "Clear Flags" to remove a flag (hot key L) or select either "Flag Not Found" or "Flag Bad" to add a flag. What's the difference between "Flag Not Found" and "Flag Bad" ? Both allow signal measurements to be recorded for that feature, however, the "bad" flag tells you (when you are doing higher-order analysis later) that the data for that spot has been deemed artifactual by the person performing the analysis; the "not found" flag tells you that the signals may not be reliable owing to spots of anomalous size/shape or very faint signal. Generally speaking, many users rely on the "not found" flag for tagging all spots that appear unreliable. Using this simplified approach, we consider the unflagged spots to be "good". Obviously, you are welcome to use the flags as you wish. However, I recommend that you NOT use the "Flag Absent" option as this results in no data being collected for that feature. The "Flag Good" option is not yet in use as the database does not yet recognize this flag as a selection criteria.

Measurements

Once you have visually inspected the feature indicators for good placement, click on the **Analyze button** (hot key Alt+A). All measurements are calculated at this step and are automatically displayed in the "Results" tab. This represents your raw data and is formatted as a *.gpr file*. The *.gpr file* and a *.jpg file* (i.e. a color JPEG image of the array) are the two files that must be saved and deposited on the database for subsequent analysis. To save these two files, press the **Save As... button** to open the "Save Results" window. Select your disk, name the file and BE SURE THE BOX AT THE BOTTOM OF THIS WINDOW IS CHECKED so that the JPEG image is automatically saved, too. Then press **Save** and the .gpr file and the .jpg file will be saved to your disk.

GenePix Inspection Tools

To visually inspect your data, GenePix Pro employs a number of useful tools. Under the "Image" tab, you can place the pointer on a spot and see the gene name and intensity information in the feature viewer at the bottom left. Under the "Scatter Plot" tab, you can select spots for viewing using the feature viewer based on signal intensity or ratio by placing the pointer on a feature in the scatter plot. You can also select one or more features in the scatter plot and they will be highlighted in the "Results" file for viewing specific measurements. Under the "Report" tab, there are a number of scripts (with instructions) that allow you to distill the array data down to the most useful information. For example, the "Interesting Genes Report" allows you to set ratio cut-offs for generating an "outlier" list of the genes with the largest calibrated ratios (calibrated 3 different ways). This list can then be exported (saved) as a *.htm file*, which can be opened in Excel. Back at your lab, you can print out the outlier list and a picture of the array, which you can keep on file. For additional information, you can check out the Axon GenePix website at **www.axon.com** or contact Axon technical support at **tech@axon.com .**

Accessing the Database for Depositing Data and Using the Web Array Tools

Our informatics partners at CIT have recently begun teaching an informatics training class designed to familiarize array users with the NCI microarray database and the online analytical tools for higher-order analysis. Once you have been trained by us at the ATC to set up hybs, scan, and analyze the arrays you can attend the array informatics class given by a CIT informatics representative. You should sign up for this class through the CIT (CIT Training Course #972), and the signup page can be directly accessed from the mAdb system home page http://nciarray.nci.nih.gov/ . During the class you will learn how to access the database, deposit array data, and utilize the tools for data interpretation and visualization (i.e. array-array comparisons, hierarchical clustering, multidimensional scaling, and clone reporting). Any future questions with concern only to the database can be emailed to: madb-support@bimas.cit.nih.gov.

With each array version that the facility prints, a new GAL file (Gene Array List - a list of genes represented on the array that defines the relative position of each element on the array) will be generated. The GAL file is uploaded into the analysis software to identify the features on your array. The naming of this file will clearly indicate which version of the arrays it refers to. GAL files can be downloaded at the NCI/DCS Microarray Database Gateway at http://nciarray.nci.nih.gov. For any questions you may have in the future, please feel free to email our staff: nciarrays-r@mail.nih.gov.

Building 10 NCI Array Center

The Building 10 NCI Array Center houses two Axon GenePix scanners and four computers in the facility, two set up for scanning and two set up for analysis only. The PCs designated for analysis are equipped with GenePix Pro 3.0 ---the same instrumentation/software that we provide at the ATC for your training and subsequent use. There is no NOVELL access from these computers, so you must bring a PC-Zip disk, or a recordable CD to save your data. The center is located in 10/4B54 and is maintained by the laboratory of Louis Staudt (NCI Metabolism Branch), located around the corner from 4B54 in 4N114. The current procedure to obtain access to the Array Center is to come by Bldg 10, Room 4N114 and sign up on the clipboard on the left hand side as you walk in. This must be done in person; they will not accept a phone call to sign you up. Each researcher can sign up for up to two hours a day of scanning time. You can access this key Monday - Friday from 9a.m. to 6p.m. with the exception of Tuesdays and Wednesdays from 12:15 p.m. to 2:00 p.m. Ask any of Dr. Staudt's lab personnel for the 4B54 key and sign-out pad.

You are also welcome to return to the main facility at the ATC (room 128) to set up your overnight hybs and wash, and/or just scan, and analyze your arrays. Most of the previously trained researchers find it convenient to hyb and wash their arrays in their own lab, and then carry them to 10/4B54 or ATC/128 for scanning and analysis.

NCI-Frederick's LMT Microarray Lab

There are two Axon GenePix scanners housed at the LMT Microarray Lab. This is located at 915 Tollhouse Road, suite 211, Frederick.

If you need to contact the NCI Microarray Center in Gaithersburg, you may call 301-435-7888.
If you need to contact personnel at the Array Center in building 10, you may call 301-496-8890.
If you need to contact personnel at LMT in Frederick, you may call 301-846-5676.

Ordering Arrays

There is a website available to members of the NCI for ordering microarrays and signing up for training. Just point your browser to - http:// arraytracker.nci.nih.gov /index.cfm The Microarray Tracker website is the only mechanism for ordering arrays (human oligo and mouse oligo) from the NCI Microarray Facility and the MTL Laboratory. Additionally, the Microarray Tracker website is used to register for the Microarray workshop.

Some of you may have set up an account when you signed up for training. If you do not yet have an account on the Microarray Tracking System it will be assumed that you have no microarray training and you will be automatically signed up for the next class before you can order arrays. If you are an experienced array user and do not need training you will have to contact nciarrays-r@mail.nih.gov to get authorization to receive an account.

Please note that in order for our system to process your order your PI must have an established Center Number in our database. A Center Number is NOT a CAN number. In order to purchase microarrays on this ordering system, users should first go to: http://arraytracker.nci.nih.gov/centercheck.cfm to check if their PI has an established center number in our system. User's who do not find their PI but do have the required information should go to: http://arraytracker.nci.nih.gov/centercheck2.cfm to fill out

the request form on that page. We will then notify your AO, and they will be responsible for assigning a center number and replying to the Array Tracker system. It will be the responsibility of your AO and PI to insure that you have a valid Center Number.

If you have any problems or suggestions feel free to e-mail the NCI Microarray Facility at nciarrays-r@mail.nih.gov . Microarrays are not a stock item, please allow 3 - 4 weeks for printing and filling orders. We are filling all the orders as quickly as possible, and you will be notified by e-mail when your arrays are ready.

FAQs

Who do I contact with questions?
The best way to get a quick answer is to use the nciarrays-r@mail.nih.gov email address. This is monitored daily and any question will be answered as soon as possible by the appropriate group or person.
If your question is about any array business (or Array Tracker) you should always email nciarrays-r@mail.nih.gov.
If your question is particular to uploading data problems on the mAdb website or to set up privileges you should contact madb-support@bimas.cit.nih.gov .

What are the microarrays composed of and how are they manufactured?
Our microarrays are composed of 70-mer oligos immobilized on Corning UltraGAPS™ coated slides. The oligos are spotted onto the slides using a GeneMachines microarrayer. The arrayer can print up to 100 arrays at a time. Arrays are typically printed in 32 or 48 sectors (or "blocks" of spots) depending on the number of printing tips used. The printing tip works much like a quill pen. It wicks up a fraction of a microliter of oligo and deposits a fraction of a nanoliter of oligo onto each slide. Each spot diameter can vary but averages ~100 um. Spots are printed with center-to-center spacing ranging from 160-220 um depending on the size of the array and density requirements. Printing with a 32-tip configuration, it takes approximately 18 hours to manufacture 100 microarrays each consisting of 22464 spots for the Operon Human Version 2.0 oligo set. Printing with a 48-tip configuration, it approximately 22 hours to manufacture 100 microarrays each consisting of 34,580 spots for the Operon Human Version 3.0 long oligo set. And printing with a 48-tip configuration, it approximately 24 hours to manufacture 100 microarrays each consisting of 38,467 spots for the Illumina Mouse MEEBO long oligo set.

How do the microarrays work?
Fluor-labeled cDNA is generated from purified RNA (mRNA or total RNA). One RNA sample is labeled with Cy3 (emits green fluorescence at 532 nm) and the other is labeled with Cy5 (emits red fluorescence at 635 nm). The labeled cDNAs are combined and hybridized to the same microarray. The cDNA hybridizes to the probes via complementary interaction. The ratio of Cy5 signal to Cy3 signal can be calculated for each spot on the array and indicates the relative ratio of gene expression. These ratios can be normalized and converted to color for visualization purposes. Traditionally, a yellow spot indicates a 1.0 (or a 1:1) expression ratio which means no difference in expression levels between the two samples while a red spot (>1.0) indicates that gene is expressed at higher levels in the sample labeled with Cy5. Conversely, a green spot (<1.0) indicates that gene is expressed at higher levels in the sample labeled with Cy3. (The expression ratio is generally reported as Cy5/Cy3, or red over green.)

How should the arrays be stored and how long do they last?
The arrays at the NCI Microarray Facility are stored at room temperature in a desiccant chamber for minimizing humidity. We recommend that the slides be stored in slide mailers, at room temperature, in a sealed container with desiccant to keep out dust and humidity. We have found that the arrays stored this way last for 6 months and still deliver optimal performance.

Where do the genes represented on the microarrays come from?
Currently, we are printing the Qiagen Human Version 2.0 set of oligos for human arrays and Compugen Mouse Release 2.0 for mouse arrays. Each company has their own proprietary bioinformatic algorithms for selection of oligos. However, oligo design and optimization criteria include incorporating alternative splicing structure by selecting probes from regions that are common to a maximal number of predicted splice variants, minimizing cross-homology, maintaining sequence quality by avoiding sequencing errors and polymorphic sites, normalizing GC content / Melting Temperature across the collection, minimizing the distance from the 3' end, avoiding oligo secondary structures, and considering genomic information.

How can I find information for an oligo on the array?
First go to the Qiagen's Oligo Microarray Database at http://oligos.qiagen.com/arrays/omad.php. Follow the link for the appropriate oligo set. Currently, we are printing Human Version 2.1 and Version 3.0.
For mouse arrays information can be found at http://www.labonweb.com/chips/libraries.html regarding the Compugen Mouse Release 2.0 oligo library. For more information on the Mouse MEEBO set visit http://www.illumina.com/products/dna/genomesets/meebo_mouse.ilmn.

How long can labeled cDNA be stored prior to hybridization?
Some users have successfully kept labeled cDNA if stored at –20ºC under dark conditions for up to one month. Store labeled cDNA without SSC, SDS and before the final denaturation step.

How soon after washing should the arrays be scanned?
It is best to scan them as soon as they are washed. As a general rule, the arrays should be scanned the same day they are washed – preferably within a few hours of washing. Be aware that during the summer months when ozone levels are high, signal decay can occur quickly.

How much total RNA is required for labeling?
For maximum signal detection and reproducibility, we recommend at least 10μg of total RNA. The amount of total RNA required for labeling is dependent on the quality of the RNA and the level of mRNA in the sample.

How do I increase my hybridization signal?
There are a number of ways to approach this question. If your signal is consistently weak, try doubling the amount of total RNA used when labeling. Often the mRNAs can account for a much smaller fraction of your total RNA sample then expected. By increasing the amount of template RNA used in the RT reaction, you can often increase the amount of labeled target. However, it should be noted that this is not always the solution. Degraded RNA is a problem that accounts for low signal as well. In some tissue types, isolating high molecular weight RNA is inherently difficult. If RNA quality is in question, we recommend running a few micrograms out on a gel or using Agilent's Bioanalyzer to check for degradation. In many cases, higher quality RNA is necessary for better array hybridizations. Also, certain reagents that go into the RT reaction can go bad overtime. DTT and dNTPs are particularly sensitive to multiple freeze/thaw cycles. Fresh RT enzyme can sometimes make a positive difference, too. New methods in probe labeling are emerging that promise to increase signal sensitivity.

How do I decrease the background signal on my array?
Background often results from high salt and detergent concentrations during the hybridization. Most often, high salt and detergent concentrations occur as the result of evaporation of the hybridization solution. Evaporation drives the salt and detergent concentrations up and may facilitate the precipitation of target onto the glass surface (particularly Cy5-labelled target). Evaporation can occur rapidly at two particular steps in the protocol: 1) when the target is first pipetted onto the array, and 2) following the hybridization when the hyb chamber is opened and the array is exposed to cold, low-humidity air. Point number 2 is potentially the most critical. Most hyb chambers have aluminum bases. When they come out of the water bath, they remain hot for several minutes. Once the cover is removed, the array is essentially sitting on a hot plate in a cold environment. At this point evaporation of the target around the edges of the coverslip can occur almost instantaneously. Therefore, you must work quickly to remove the array from the hyb chamber and submerge it in the first wash buffer.

Sometimes the background actually results from fluorescence emanating from the opposite side of the array. We routinely clean the backside of the arrays (after washing and drying) with moist breath and a Kim wipe – just prior to scanning. In cases of particularly weak signal, we often have to scan at near-maximum PMT voltage that results in relatively high background levels. To combat this phenomenon, you should try to increase signal (i.e. probe intensity) and scan at lower PMT voltage.

It has been reported that organics used during RNA extraction can contaminate RNA samples and contribute to background fluorescence. Likewise, benzene which is used to dehydrate 95% EtOH to 100% EtOH, has been implicated in background artifacts. For this reason, many researchers avoid using EtOH preparations made with 100% (200 proof) EtOH.
We have seen that re-soaking the arrays in each wash buffer for several minutes can reduce some forms of high background. Specific signal can remain while background diminishes.

How do I get rid of the "scars" that I occasionally see on my arrays?
Sometimes we observe symmetrical and parallel scratches on arrays that would seem to indicate mechanical forces acting on the surface. The most likely culprit is the coverslip. To avoid coverslip scratching, try a more gentle approach when removing the coverslip. We recommend inverting the array (coverslip down) in the first wash buffer (containing SDS which helps loosen the coverslip) and gentle rocking up and down until it starts to move. Then slowly tilt downward and let the coverslip fall away on its own volition.

How do I get rid of all those tiny "specks" of fluorescence I see on my arrays?
We call this speckling "schmutz" and it comes in a variety of flavors. It can be particulate from the air (such as dust and lint fibers) that settles onto the array surface or into the target during preparation and binds to the array surface during the hybridization. It can be precipitate such as SDS or cDNA aggregates that result from aberrant salt and detergent concentrations or unknown contaminants. Sometimes this phenomenon seems to be associated with a particular sample of

RNA, which can be corrected by RNA purification. If this phenomenon occurs with frequency, you can clean the target on a 0.20-0.45 um filter, which retains the particulates on the surface and allows the probe to pass through.

How do I minimize the "dim signal" areas on my array?
The dim areas often have a circular boarder suggesting a bubble is the culprit (although obvious bubbles do not always result in dim areas). Though we try to minimize bubble formation when applying the coverslip, bubbles that were not initially apparent can form during the hybridization as a result of de-gassing. Sometimes the dim areas display a pronounced signal gradient suggesting nonuniformity of hybridization stringency across the surface of the array. We and others think that this phenomenon arises due to initial volume gradients under the coverslip. Volume gradients can result from warped coverslips and uneven distribution of probe beneath the coverslip. When applying a standard coverslip to the hybridization solution (pipetted onto the array surface), we recommend a centered and level release such that the target spreads beneath the coverslip in a radial fashion converging on all four corners of the coverslip simultaneously. Coverslips that possess "vertical lifts" on two or more edges can potentially reduce volume gradients and eliminate dim areas.

How do I just get better array results?
Think critically about what you are doing when you are isolating your RNA and making your probe. Quality is everything when it comes to microarray science. Pure, high-molecular weight RNA is absolutely required for good array results. Fresh reagents and meticulous technique can make the difference between good probe and no probe. Be observant every step along the way and always consider the molecular and physical consequences with each manipulation of your RNA, labeled cDNA, and the array or coverslip. Everyone has a learning curve, which usually begins with consistently sub-average hyb outcomes and culminates in consistently stellar hyb outcomes and array data. All it takes is a little patience and a lot of critical thinking.

Some Commercial RNA Amplification Kits:
Many amplification kits/protocols have come to market within the past year.

1. Ambion http://www.ambion.com/catalog/SubApps.html?4
2. Acrturus http://www.arctur.com/research_portal/products/
3. Genisphere http://www.genisphere.com/
4. Nugen http://www.nugentechnologies.com/
5. Epicentre http://www.epicentre.com/item.asp?ID=454&CatID=12&SubCatID=26

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