

Scan slides (Axon Genepix 4200AL)

We need to scan the slides on both channels (Cy3 and Cy5) to obtain a 16-bit grayscale TIFF file for each. Typically these files are about 20-26Mb per channel, where the arrays cover most of the slide. Genepix, and other software, allows you to save a single file containing both images. I prefer to save the single images separately, as this allows me to use any software for quantitation, not just Genepix. The process indicated here is specific for an Axon Genepix 4200AL scanner, but it is essentially the same process for any other laser scanner.

- Turn on scanner (it needs about 15min to warm up the lasers, you will not be able to scan until the lasers are ready).
- Turn on the computer and start Genepix 6.0

First we will do a preview scan of each slide, to adjust the PMT settings and set the area to scan, we will save those settings into a .GPS file, and afterwards we will set the scanner to scan all the slides with those settings.

- Select `Batch Scan` tab.
- Load slides into cartridge, with the spotted side towards you, and the label on the left (you can put the label on the right too, but if on the left, the slide will appear on the screen upright with label at the bottom, which is the usual way to look at them).
- Load cartridge.
- Adjust global settings (click on  on the right panel). This probably only needs to be done the first time, if you save your own personal profile in Genepix, but it's good to check just in case.

Most defaults are good, but I'd change these:

File Save

Image and results file naming: `untick all, except perhaps "barcode" if your slides have one, and can be read by the scanner (some barcodes won't be read!)`

Save as type

`Single image TIFF files`

`Do not use LZW compression`

Folders

`Select the appropriate folders in which to save the data.`

Workflow

Untick “search for a barcode...” if you do not intend to use that feature, as it adds extra time between scans.

Acquisition, automatically... Tick all, to register (align images from both channels properly) and save images after scan, and to eject slide carrier after the batch scan is finished.

Alignment

Align features: Find irregular features

Preview scans

- Scan type / single
- Select first slide to preview on the mapper, click Select.
- Open the hardware settings window on the right panel: 
- Choose the channels to use, and the order (635 for Cy5 and 532 for Cy3), and the right filters. This is probably already correct by default if attached to a scanner. But you may change the order of the scans, and whether to preview just one channel instead of both.
- Enter laser power. 75% is usually more than enough.
- Enter PMT settings. This we will adjust during the preview, but start with a low setting, such as 300-400.

The higher the laser power, the brighter the direct signal (and also the stronger the photobleaching effect), resulting in requiring a low PMT (optical photomultiplier) setting, and low noise. The PMT response is a sigmoidal curve, where the linear range is achieved in the middle, so avoid extremely low or high values for PMT. The manual recommends PMT values of 500-900, but I am finding that with some very bright slides it is difficult to achieve PMT settings above 400-500 without saturation, at laser powers as low as 30%!!!

We want to produce usually several scans from the same slide, as it is difficult to use the whole dynamic range effectively from just one scan. The first scan will aim to get no or just a few saturated spots. A second scan will be made so that the weakest spots show a decent level, even if the number of saturated spots will be large. Additional scans may be made.

- Hit the preview button on the right panel: 
- Go to the Image tab and zoom in as appropriate. Observe the image produced as the preview scan proceeds. Adjust the PMT setting as high as possible avoiding excessive saturation.
- Repeat as required for the same channel or both.
- The Histogram tab shows a histogram of the number of counts and their intensity (how many pixels of each intensity) for each channel. Ideally we would like to balance both channels so that the ratio is close to 1. As we assume that most genes show no differential expression, I tend to do most of the balancing by eye under the Image tab, judging the “yellowness” of the signal (red + green = yellow), and the histogram is usually quite close.
- The area to be scanned (only effective for the real scan, not preview) can be selected by clicking on  on the left panel, and adjusting the square as required.
- Once satisfied with the area and PMT settings, save them in a settings (GPS) file.

Save settings as...

I name the files as SlideNumber-ScanNumber.GPS (i.e: 979-1.GPS) where the scan numbers tend to be 1 (avoiding saturation), 2 (stronger scan), 3 (even stronger) etc. But that's just my system. Use whatever naming system yo wish, of course.

- Eject cartridge (unfortunately we need to eject everytime we want to select a new slide! silly design, but that's how it works...)
- Load cartridge again, and repeat PMT adjustment etc for next slide.

When all the preview scans are done and their GPS files saved, we can proceed to set up the batch scan. (If only one scan needs to be done, then proceed directly to data scan with the current settings by pressing the data scan button on the panel on the right.)

Data scan: batch scan

- Eject cartridge and reload.
- Go to `Batch scan` tab, select `Batch scan`.
- If there is any information displayed that does not correspond to our slides, clear it.
- Select a slide in the mapper or the window on the right.
- In 5b on the left, fill in the number of the slide where it says "file name body" (For instance 779-1).
- In 5c select `GPS file`, and browse to locate the corresponding settings file (i.e: 779-1.gps).
- If multiple scans of the same slide are going to be made, in 4b select how many. This will add extra versions of the slide in the window on the right, and fill in the appropriate file name body (779-2, or 779-3, etc) and in 5c select their GPS files.
- in 5a select `Scans/data scan only`.
- You can untick 6. We will do the analysis with GenePix when we choose to.
- Save these settings in as an autoloader file (GPA): click on 7.
- Click on 8: start batch scan.
- After the scans are done, they are saved automatically in the specified folder. Ready for segmentation and quantitation.

Segmentation and quantification with GenePix 6.0

- Open images
- Open array list: select GAL file containing spot ID, location, names etc.
- Find layout and define spots:



Click on  to open the spot finding menu.

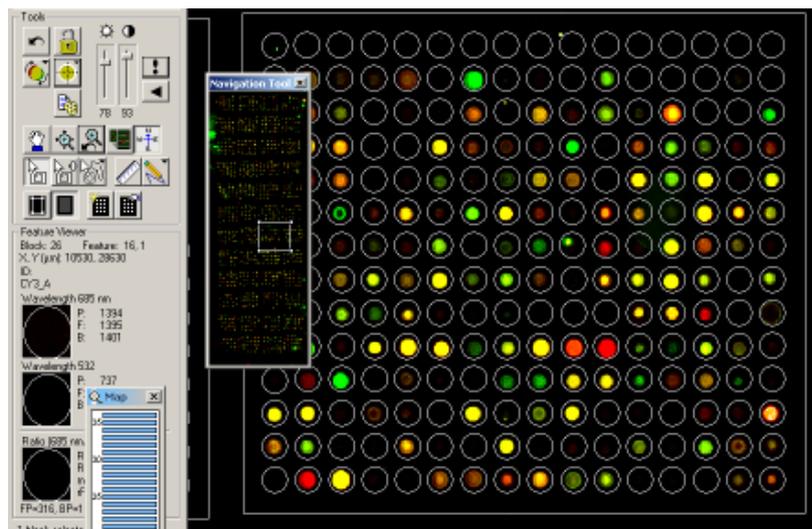
Find array locates roughly the layout of the whole slide. It takes a few seconds.

Find blocks locates the individual blocks, and it's usually good at it, but for some layouts it fails, for instance if there is a row of spots that are largely empty at either end of a block.

Check, and manually correct if required, placing the blocks in the right location (roughly, no need to bother about how accurately the spots are found at this stage).

If you have to correct manually it may take up to 2-3 minutes, max. If the blocks are accurately and consistently found for your array, then you may go for the whole automatic finding function (press F8).

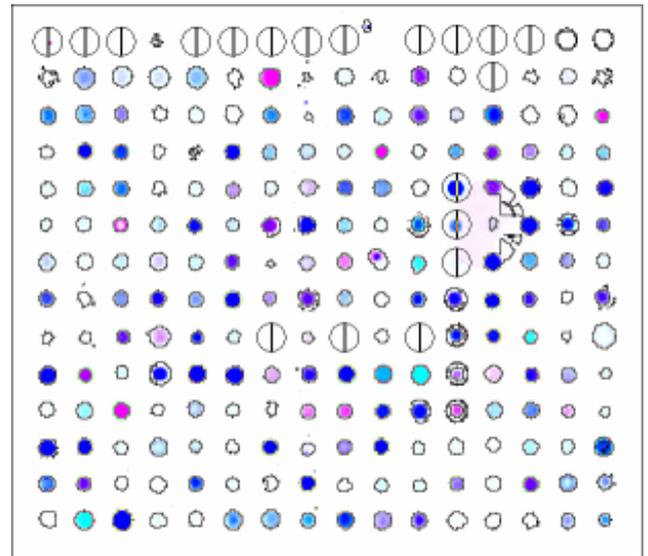
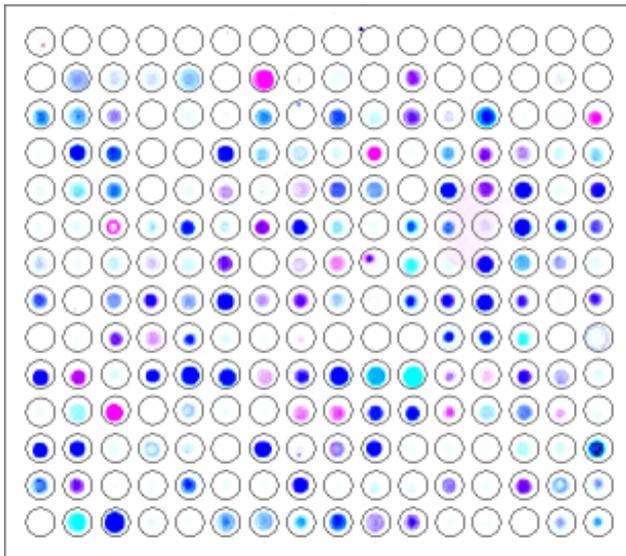
However, since correct identification of each spot is crucial, I'd rather take an extra couple of minutes per slide and make sure that everything is okay.





Use the navigator tool window to browse across the the slide quickly, checking the blocks are located correctly.

Once the blocks are positioned, click on  again, and select `Align features` in all blocks. GenePix will find the spots, and if “Find irregular features” was selected, it will adjust the size and shape of the spots automatically, and flag empty spots.



Before aligning with “irregular features”.

Afterwards.

- Save settings as... This will be a new GPS file, I *think* that it saves the laser settings etc as well, as that info is located within a header in the TIFF files, but I am not sure so I have to check! If it saves that info, it can easily just replace the previous GPS file.
- Save results as... (for instance 779-1.GPR)
- The un-normalised data can then be fed onto Limma for analysis.
- Alternatively it can be normalised within GenePix, but the options offered are not as good as those in Limma.
- If using Acuity or a similar analysis program, it's best to leave normalisation to them, as they can do Lowess normalisation and more.