Fluorescent microarray imaging and analysis

Typhoon Variable Mode Imager

Key words: microarray, gene expression, fluorescence, imaging, Typhoon, CyDyes.

DNA microarray technology is a powerful tool to investigate global changes in gene expression of cells and tissues (1). In expression profiling experiments using cDNA microarrays, thousands of discrete DNA sequences are robotically spotted onto a glass microarray slide and subsequently hybridized to fluorescently labelled cDNAs. Differential expression profiling is often used to compare the gene expression patterns of two different samples, such as diseased versus normal or drug-treated versus control. This is usually done by labelling the cDNAs from two individual samples with two different fluorescent dyes, such as Cy™3 and Cy5, followed by hybridizing the labelled cDNAs onto the microarray slides. After hybridization, the slide can be imaged by fluorescence detection. The normalized ratio of the fluorescence intensities of the two dyes is then calculated and used to determine the relative gene expression from the two samples for each spot.

The Typhoon™ Variable Mode Imager is a well proven instrument providing high sensitivity and wide linear dynamic range (five orders of magnitude) for multicolor fluorescence imaging of gels and blots (2). This application note introduces a new feature on Typhoon, the microarray imaging capability, which is available on the Typhoon 9210 and 9410 models. The Typhoon 9210 is equipped with green (532 nm) and red (633 nm) lasers. The Typhoon 9410 has an additional blue laser with two laser lines (457 nm and 488 nm).

Products used

Typhoon 9210	63-0038-51
Typhoon 9410	63-0038-55
ArrayVision™	63-0008-18
Cy3-dCTP	PA53021
Cy5-dCTP	PA55021
CyScribe™ First Strand cDNA Labelling Kit	RPN6200
HEPES	US16926
Humid hybridization cabinet for microarrays	RPK0176
Hybridization oven/shaker	RPN2511
Lucidea™ Microarray ScoreCard™ Kit v.1.1	RPK1161
Lucidea Universal ScoreCard	63-0042-85
Microarray hybridization solution	RPK0325
RNase-Free water	US70783
Sodium dodecyl sulphate (SDS)	17-1313-01
SSC - 20×	US19629

Other materials required

- CMT-GAPS microarray slide (Corning)
- Compressed nitrogen
- Human liver and skeletal muscle mRNA (Clontech)
- Microarray cover slips
- QIAquick PCR* Purification Kit (Qiagen)
- SpeedVac™ centrifuge
- UV-vis Spectrophotometer
- Water bath

*See licensing information

Amersham Biosciences

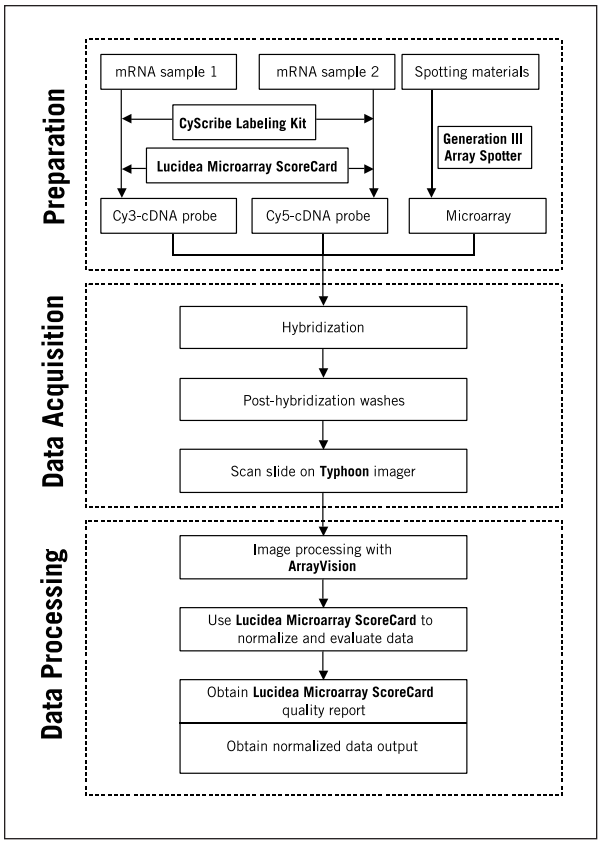

Fig 1. The overall workflow of a microarray application.

Protocol

An example of a microarray application and the preparation protocol are described in this application note. Figure 1 shows the overall workflow for preparation, data acquisition and data analysis for this application. The key products available from Amersham Biosciences used in this process are highlighted. Typhoon will also image microarray slides prepared with alternative protocols and reagents. Please refer to the manufacturer's instructions for experimental details.

For this application, the microarray slides used were robotically spotted with a Generation III Array Spotter from Amersham Biosciences. Lucidea Microarray ScoreCard Kit v.1.1 (Lucidea Microarray ScoreCard) reagents and software were used for data validation and normalization. The kit is compatible with human microarray slides spotted with a Generation III Array Spotter. A set of 4224 human genes and Lucidea Microarray ScoreCard control reagents were spotted in duplicate on a Corning CMT-GAPS microarray slide.

For non-human and human microarray slides prepared by other types of array spotters, Lucidea Universal ScoreCard reagents are recommended as universal references for validating and normalizing microarray data. The product only contains reagents and is independent of experimental platform and data analysis software. The Lucidea Universal ScoreCard reagents display no cross-hybridization over a wide range of biological species.

For more details about the Lucidea Microarray ScoreCard reagents and software as well as Lucidea Universal ScoreCard reagents, refer to the *Lucidea Microarray ScoreCard* and *Lucidea Universal ScoreCard* data files and user manuals.

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cDNA labelling

For this application, CyScribe First Strand cDNA Labelling (CyScribe) Kit was used for cDNA labelling.

1.1. Add Lucidea Microarray ScoreCard spike mix to 1 µg of the human skeletal muscle mRNA and 1 µg of the human liver mRNA samples.

1.2. Use the CyScribe Kit to label the cDNAs according to the kit instructions. Cy3- and Cy5-dCTPs are used to label skeletal muscle and liver cDNAs, respectively.

1.3. Purify the Cy3- and Cy5-labelled cDNAs using the QIAQuick PCR Purification Kit following the manufacturer's protocol.

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Hybridization preparation

2.1. For each slide, combine 30 pmol (concentration measured on a spectrophotometer) of Cy3- and 30 pmol of Cy5-labelled cDNA into one tube and dry them down in a Speed-Vac or other centrifugal vacuum concentrator. Protect the solution from light.

2.2. For each slide, clean a cover slip by spraying it with 70% ethanol. Use a lint-free laboratory wipe to dry the cover slips, and then blow them completely dry with compressed nitrogen.

2.3. Resuspend the dried, labelled cDNAs in 6 µl of RNase-Free water. Add 7.5 µl 4× Amersham Biosciences microarray hybridization solution, 15 µl 100% formamide, and 1.5 µl PolyA (1 µg/µl). The final volume is 30 µl per slide.

2.4. Denature the labelled cDNAs at 94 °C for 2 min and spin for 30 sec.

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Hybridization — Option A with a hybridization cabinet and oven

3.1. Pipette 30 µl of the labelled-cDNA solution on an area of the slide that does not contain any spots. Place the clean cover slip on the slide. Make sure bubbles are not trapped under the cover slip and that all of the arrayed area of the slide is covered with hybridization buffer.

3.2. Place the slide in a humid hybridization cabinet and place the cabinet in an incubator at 42 °C for 12–18 h.

Hybridization — Option B without a hybridization cabinet and oven

If a humid hybridization cabinet and oven is not available, an alternative hybridization method is described below.

3.1. Perform the hybridization as in Option A 3.1.

3.2. Place the slide in a humid hybridization chamber (a slide mailer with 50 µl of RNase-Free water in the bottom can serve as an adequate humid hybridization chamber). Seal the chamber with parafilm.

3.3. Incubate the slide (still in the sealed hybridization chamber) for 12–18 h in a 42 °C water bath. Protect the chamber from light.

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Post-hybridization washes

4.1. Preheat a solution of 2× SSC, 0.1% SDS (Buffer 1) and a solution of 1× SSC, 0.1% SDS (Buffer 2) to 55 °C.

4.2. Immerse the slide in Buffer 1 at 55 °C and remove the cover slip. Transfer the slide to a cradle in a staining jar containing Buffer 1 at 55 °C. Wash the slides in 55 °C Buffer 1 and then 55 °C Buffer 2 for 5 min each with gentle rocking at room temperature. Cover the staining jar with aluminum foil to prevent photobleaching of the dyes.

4.3. Fill three staining jars with 0.1× SSC at room temperature and dip the cradle into these three jars for 5 sec each. Do not allow the slides to dry between washes.

4.4. Use a low-speed centrifuge that accommodates microarray slides to spin-dry the slides. Place a dry paper towel on the plate holder to absorb the excess liquid, and then place the cradle with washed slides on the plate holder. Balance the rotor and spin for 1 min at <500 g.

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Imaging the microarray slide

For complete instructions on how to image microarray slides on Typhoon, please refer to the *Microarray Slide Holder Kit Instructions* and *Typhoon Instrument Guide*.

5.1. Positioning the slide holder and microarray slide(s)

5.1.1. Carefully clean the back of the slides to remove any liquid marks, finger prints and dust particles. Clean the Typhoon platen and microarray slide holder.

5.1.2. Gently position the Typhoon microarray slide holder at the front left corner of the clean glass platen (Fig 2).

5.1.3. Gently place the two slides into the wells of the slide holder with the spotted side face down. If scanning only one slide, position the slide in the first well (closest to A2) to minimize the scan time.

5.1.4. Make sure the slides are seated in the grooves of the slide holder and the slide holder is seated firmly against the front left corner of the platen area. The correct positions of the slides and the slide holder keep the slides at the appropriate angle and location for proper scanning.

5.1.5. Correctly position a slide restraint over each slide.

5.2. Setting up Typhoon Scanner Control

5.2.1. In the Typhoon Scanner Control window, select the following parameters:

Scan area	A2 through D2 inclusive for one slide A2 through J2 inclusive for two slides
Acquisition mode	Fluorescence
Orientation*	Я
Press sample†	On (box is checked)
Pixel size	10-µm
Focal plane	+3 mm

*If the workstation has difficulty performing a scan with this orientation, try to perform a scan with orientation ∝ and change the image orientation during the image processing step (6.1).

†The sample is pressed to prevent movement during a scan.

5.2.2. In the Fluorescence Setup window, select the appropriate laser/emission filter settings. If imaging Cy3 and Cy5, choose the settings as shown in Table 1. Select Normal for Sensitivity. Set an appropriate PMT voltage setting (the recommended range is between 450–800 V). ***For quantitative results, select Sensitivity mode to perform single channel scans.***

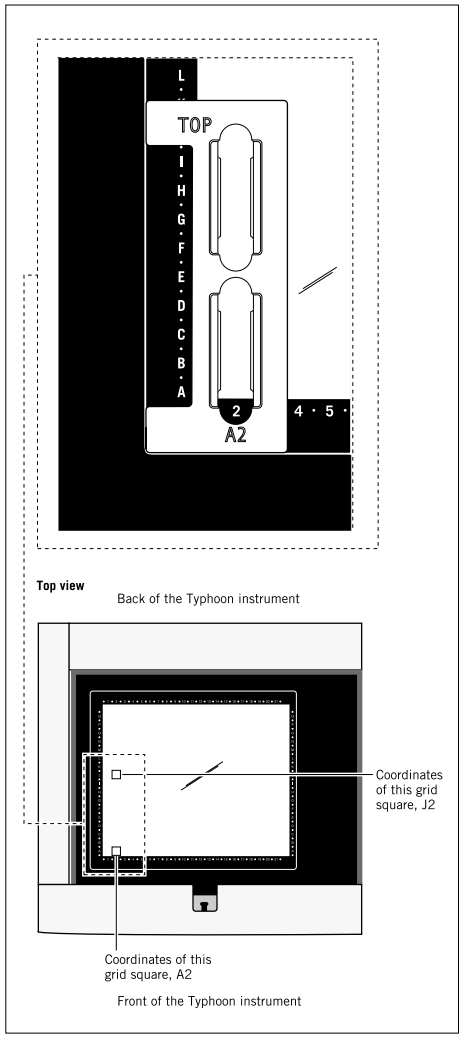

Fig 2. Positioning the microarray slide holder on the glass platen.

Table 1. Typhoon Scanner Control settings for Cy3 and Cy5 detection. The excitation and emission maxima of Cy3 and Cy5 are shown in parentheses along with their appropriate laser and emission filter selections.

Fluorochrome (Ex, Em)	Laser	Emission Filter
Cy3 (550 nm, 570 nm)	Green (532 nm)	580 BP 30
Cy5 (649 nm, 670 nm)	Red (633 nm)	670 BP 30

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Image Processing

The following procedures are recommended for image processing:

6.1. Use ImageQuant™ Tools Utility 2.2 (IQ Tools) to open and process the Typhoon microarray images. After the images are opened, make sure the image orientation is appropriate for analysis using either the bar code or control spots on the slide as landmarks. If necessary, change the image orientation using the rotating or flipping tools in IQ Tools.

6.1.1. If the image contains two slides, use the cropping tool to crop out each slide and use Save Region of Interest As to create separate image files for them.

6.1.2. If Lucidea Microarray ScoreCard is used for data validation and normalization, prepare the image files by cropping the left and right half of the image and use Save Region of Interest As to create individual half-image files. It is recommended to name the files as:

SlideID_left.ds
SlideID_right.ds

The half-images are now saved in these folders:
SlideID_left.DIR
SlideID_right.DIR

Each folder contains these image files:
UNSEP1.gel (channel 1)
UNSEP2.gel (channel 2)

6.2. Use ArrayVision version 5.1 or higher for image quantitation. The images processed with ArrayVision are compatible with Lucidea Microarray ScoreCard.

For more details, refer to the *ImageQuant Utilities, Lucidea Microarray ScoreCard,* and *ArrayVision* user manuals.

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Data Validation and Normalization

For the application described in this note, Lucidea Microarray ScoreCard was used for data validation and normalization. For more details, refer to the *Lucidea Microarray ScoreCard* user manual.

Results

Labelled cDNA from human skeletal muscle and liver were hybridized onto a slide spotted with 4224 human genes in duplicate plus the Lucidea Microarray Scorecard controls in 24 replicate. After washing, the hybridized slide was scanned on a Typhoon 9410. The spots were about 200 µm in diameter.

To validate the microarray experiment, it is important that the variations in the measurements are evaluated so that accurate comparisons can be made within an experiment and across multiple experiments. This is a common requirement with any quantitative microarray application. In this application note, data validation and normalization is accomplished using the Lucidea Microarray ScoreCard controls and software. The Lucidea Microarray ScoreCard controls include negative, dynamic range, and ratio controls for Cy3 and Cy5. Negative controls are used to evaluate the degree of nonspecific hybridization and provide the detection threshold value. Dynamic range controls are mainly used to estimate detection limits and linear range. Ratio controls provide a mechanism for verifying the accuracy of calculated gene expression ratios. Table 2 illustrates the concentrations and relative abundance in the labelled sample of the dynamic range control and ratio control elements.

Table 2. The relative concentration and abundance of Lucidea Microarray ScoreCard dynamic range and ratio controls. RC and DR stand for ratio control and dynamic range control, respectively.

Control sample	Cy3:Cy5 ratio	Conc in mix (pg/5µl mix) Cy3	Cy5	Relative abundance
1DR	1:1	33 000	33 000	3.30%
2DR	1:1	10 000	10 000	1%
3DR	1:1	1 000	1 000	0.10%
4DR	1:1	330	330	0.03%
5DR	1:1	100	100	0.01%
6DR	1:1	33	33	0.003%
1RC	1:3	1 000	3 000	NA
2RC	3:1	3 000	1 000	NA
3RC	1:10	1 000	10 000	NA
4RC	10:1	10 000	1 000	NA

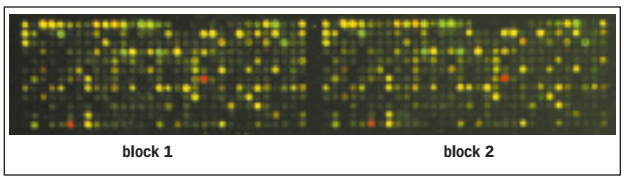

Fig 3. A section of a microarray slide imaged on a Typhoon 9410. Human tissue cDNA from skeletal muscle (labelled with Cy3, shown in green) and cDNA from liver (labelled with Cy5, shown in red) were mixed and hybridized onto a spotted slide with 4224 human genes printed in duplicate. Shown here are two (left and right) duplicate blocks out of a total of 24 blocks of spots on the whole slide. Row 1 of each block contains the set of Lucidea Microarray ScoreCard controls (32 spots). Rows 2 through 12 contain a duplicate set of 352 human genes.

To evaluate the detection limits and dynamic range of this application, six dynamic range-control samples were used. The sixth dynamic range control (6DR) has the least relative abundance of 0.0033% (33 pg per µg of sample mRNA). Even at this low relative abundance level, the signal-to-noise ratios for Cy3 and Cy5 detection for 6DR were determined to be 5 and 13 respectively, demonstrating Typhoon's high sensitivity for both Cy3 and Cy5 spot detection. Typhoon's limit of detection (LOD) was determined to be 0.002% in relative abundance level (20 pg per µg of sample mRNA) for imaging Cy3 and 0.0008% (8 pg per µg of sample mRNA) for Cy5. The LOD was determined by converting the fluorescence intensity level of the detection limit at which the background-corrected signal-to-noise ratio is 3 to the corresponding abundance level.

The signals were determined to be linear at least to 1% (10 000 pg) in relative abundance for both Cy3 and Cy5 detection. For higher abundance genes (such as 3% or 33 000 pg), the DNA on the microarray spot was hybridized to saturation causing the fluorescence intensity to plateau. Please note that Typhoon has a wide linear dynamic range of 5 orders of magnitude (from count 1 to 100 000). Typically, the range of microarray gene expression levels is limited to 2.5 to 3.5 orders of magnitude.

To determine the relative gene expression from two samples, the ratio of the fluorescence intensities of Cy3 and Cy5 needs to be evaluated and normalized. The normalization process of the Lucidea Microarray ScoreCard software is designed to correct for the difference in Cy3 and Cy5 fluorescence intensities which are caused by factors other than differential gene expression, such as variations in Cy3- and Cy5-dye incorporation during labelling and Cy3 and Cy5 imaging sensitivity. Table 3 shows the result of the ratio analysis on the ratio and dynamic range controls before and after normalization. In general, the normalization procedure brings the Cy3/Cy5 ratio much closer to the expected value than the observed (unnormalized) value.

Table 3. Lucidea Microarray ScoreCard ratio analysis (Cy3/Cy5). RC and DR stand for ratio control and dynamic range control, respectively.

Control (Cy3/Cy5)	Expected	Observed	Normalized
1RC (1:3)	0.33	0.73	0.31
2RC (3:1)	3	7.8	3.2
3RC (1:10)	0.1	0.28	0.12
4RC (10:1)	10	21	8.6
1DR (1:1)	1	2.2	0.97
2DR (1:1)	1	2.2	0.96
3DR (1:1)	1	2.5	1.0
4DR (1:1)	1	2.6	1.1
5DR (1:1)	1	2.6	0.95
6DR (1:1)	1	3.0	1.1

Conclusion

The 10-µm pixel size option on Typhoon allows high-resolution scanning suitable for microarray applications. Typhoon offers high sensitivity for successful detection of genes at very low abundance levels. The wide range of excitation sources and emission filters are suitable for multicolor microarray imaging. Typhoon also provides flexibility for imaging a variety of fluorescent labels in addition to Cy3 and Cy5. The Typhoon microarray images can be analyzed with spot finding software such as ArrayVision.

References

1. Brown, P.O. and Botstein D., *Nat. Genet.* **21** (1 Suppl), 33–7 (1999).

2. *Fluorescence Imaging: principles and methods*, Amersham Biosciences, code number 63-0035-28, (2000).

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