

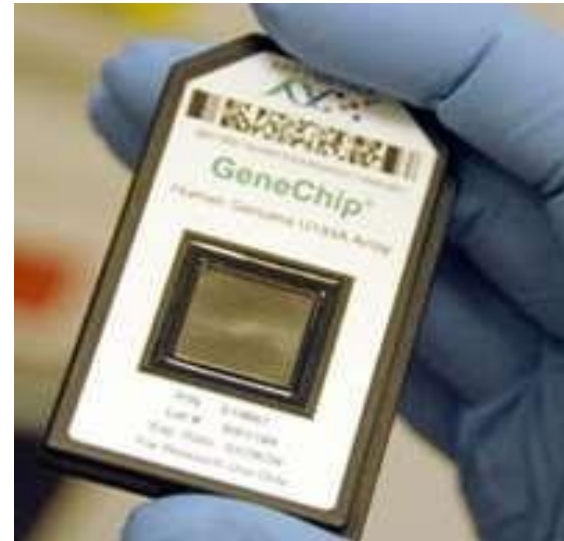
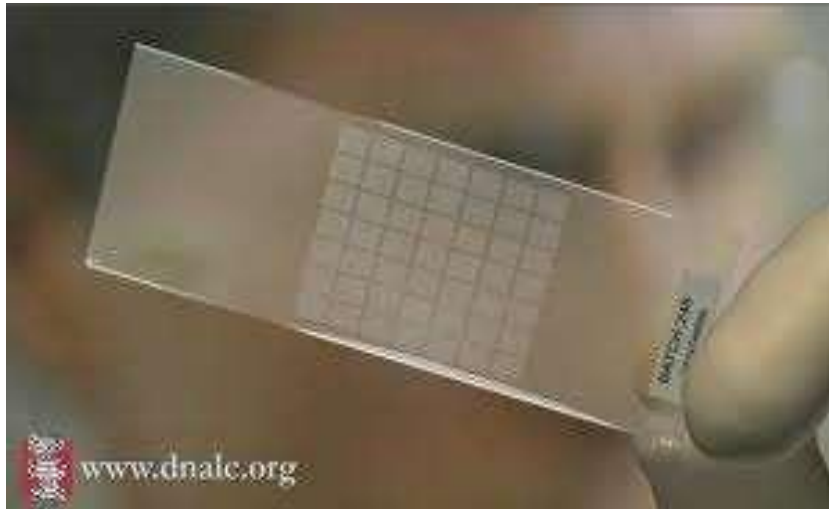
Microarray Technology and Applications

Purnima Kartha. N

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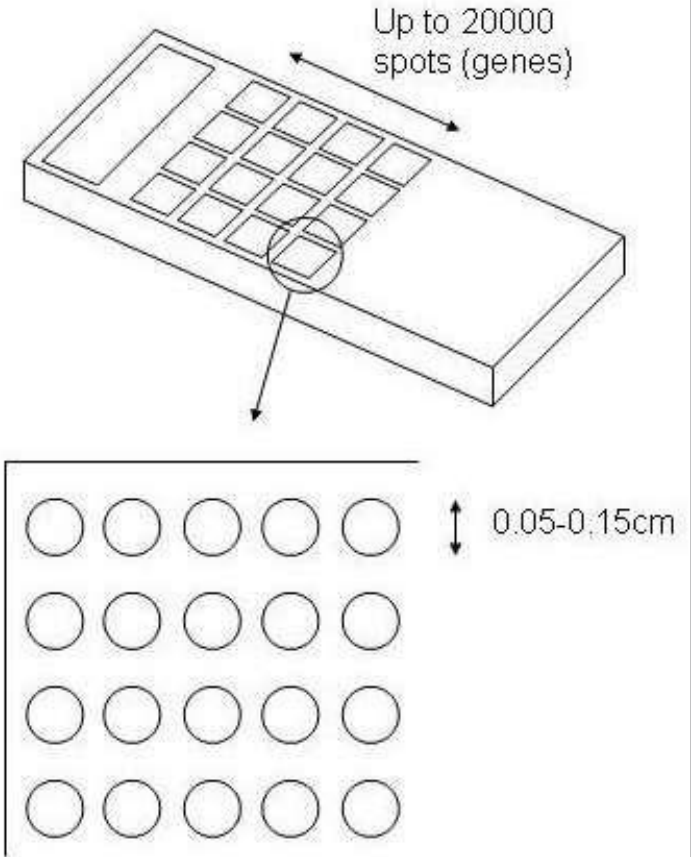
INTRODUCTION

- A microarray consists of a solid surface to which biological molecules are arranged in a regular pattern.
- Applicable in the fields of DNA, proteins, peptides and small molecules like metabolites and drugs.



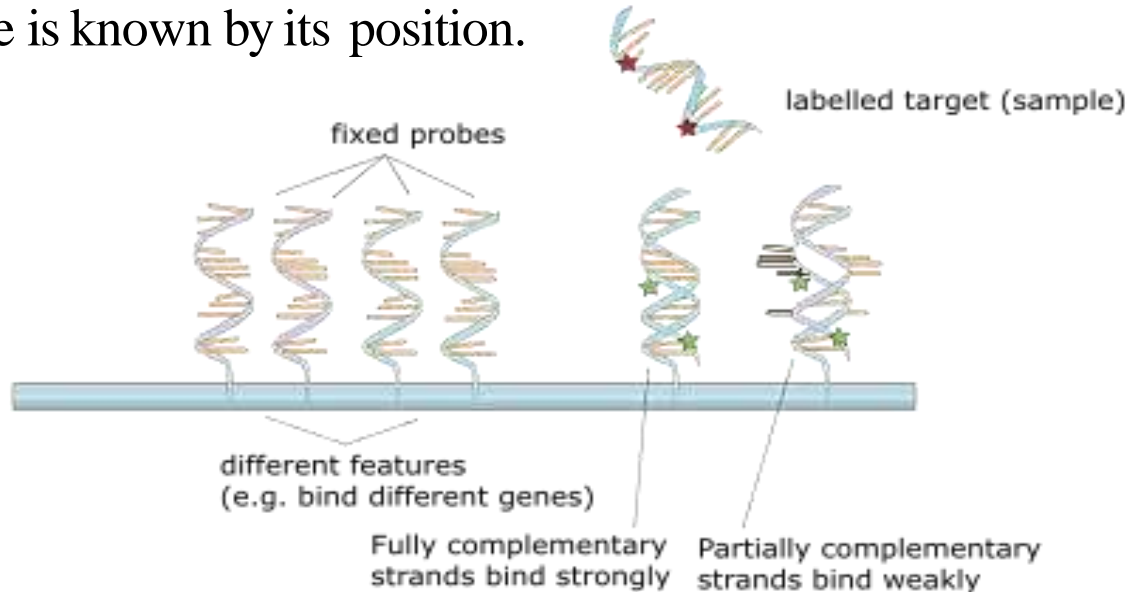
DNA MICROARRAY

- Orderly arrangement of thousands of identified sequenced genes printed on an impermeable solid support, usually glass, silicon chips or nylon membrane.
- Thousands of spots each representing a single gene and collectively the entire genome of an organism.
- Measurement of Gene Expression.



PRINCIPLE

- **Hybridization** between two DNA strands
- Microarrays use **relative quantitation** : Intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

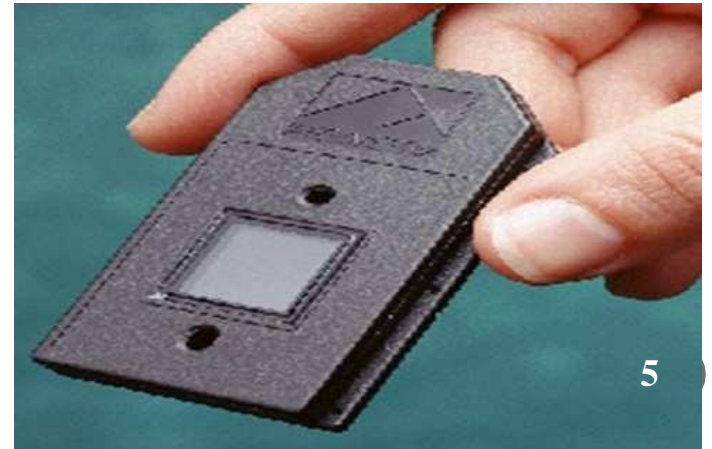


TYPES OF MICROARRAYS

1. Glass DNA microarrays which involves the micro spotting of pre-fabricated cDNA fragments on a glass slide.

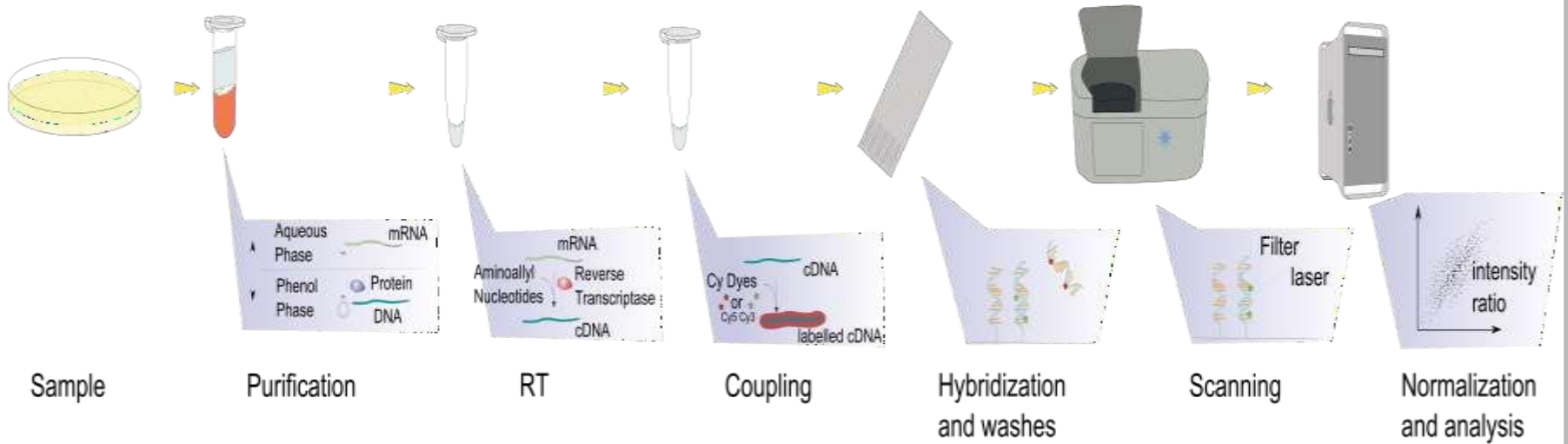
2. High-density oligonucleotide microarrays often referred to as a "chip" which involves *in situ* oligonucleotide synthesis.

- Affymetrix : by photolithography
- Agilent: Inkjet printing technology



STEPS

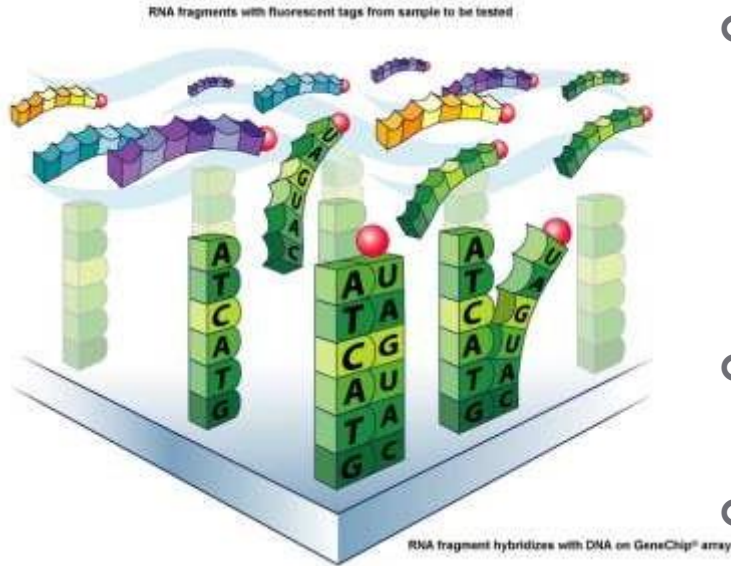
- The experimental steps involved include:



1. ARRAY PRINTING

- The microarray slide is uniformly coated with a chemical compound that will interact with and immobilize nucleic acids, irreversibly binding them to the surface.
- Nucleic acids are deposited on the slide by contact printing, and treated with UV light or baking at 80°C to crosslink them to the slide surface.
- The printed slides are stored desiccated at room temperature, in the dark, until required for experimentation.

PROBES



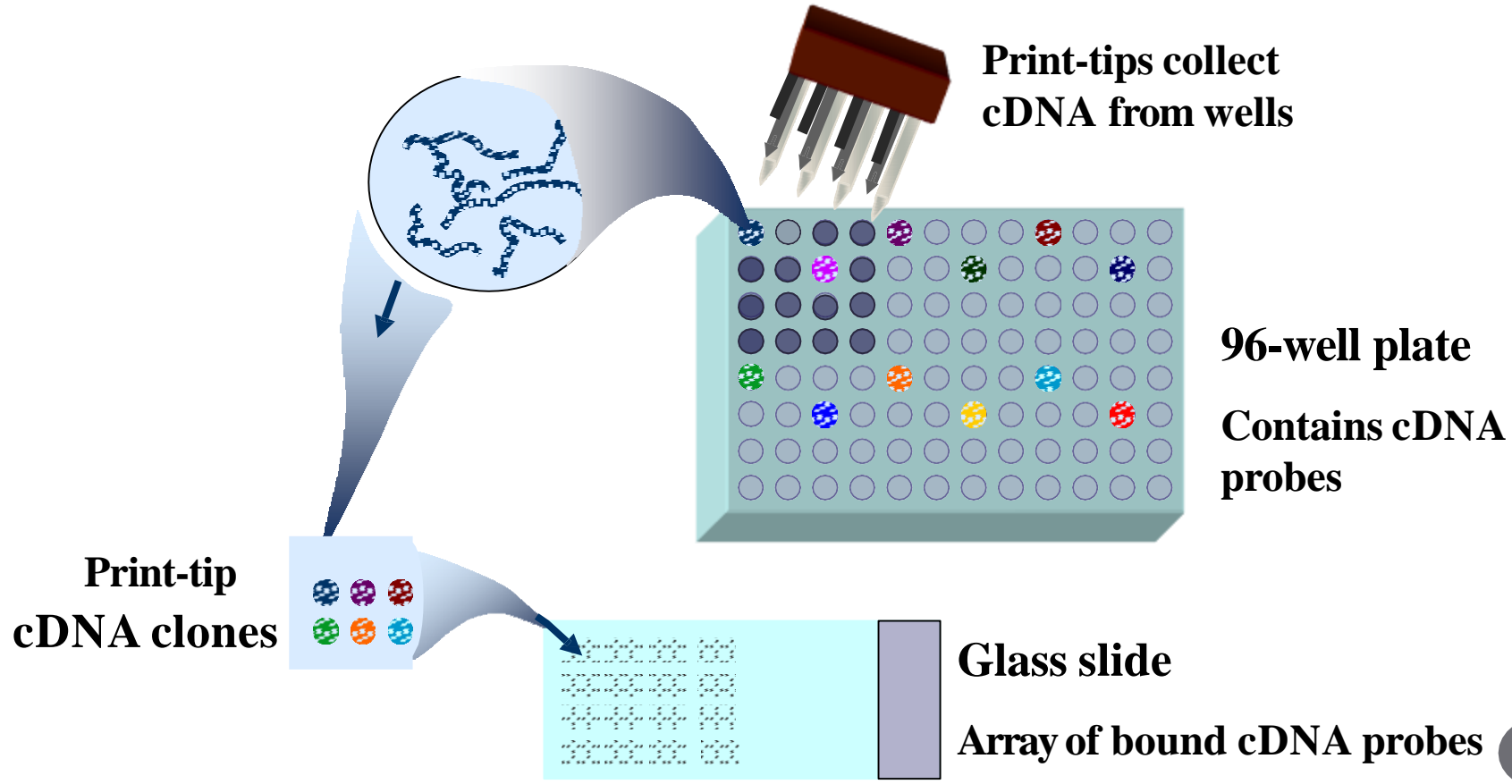
- The selection of probe sequences for a microarray design depends upon the use envisaged for the array.
- Specific probes can be designed against genes, transcripts or portions of transcripts.
 1. Clone sets
 2. cDNA library preparation
 3. Specific DNAs amplified.
- All clones purified by gel filtration/ precipitation to reduce unwanted salts.
- Selection of probes requires balancing of 4 criteria during their production: Sensitivity, Specificity, Noise, and Bias.

Printing PCR products onto glass slides:

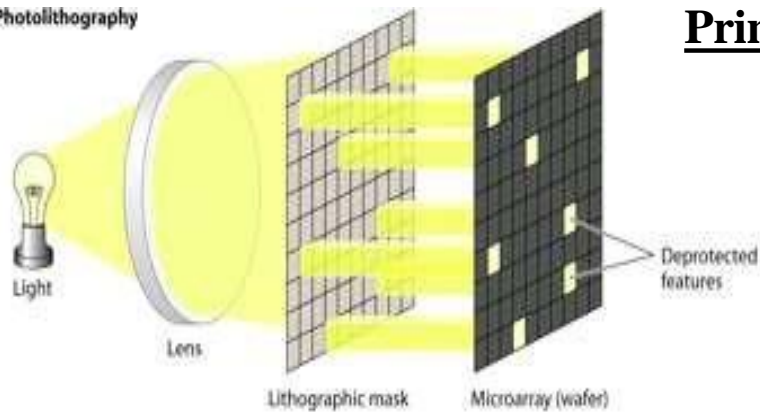
- Printing involves the sequential transfer of individual PCR products from the plates to defined areas of glass slides.
- Glass slides pre-coated with **poly lysine, amino silanes or amino-reactive silanes** - to increase the hydrophobicity of the slide, improving the adherence of the deposited DNA and minimising spreading.
- Few nLs of each DNA is deposited onto each slide, resulting in formation of spots of 50-150 μ M diameter.



Printing of cDNA microarrays



Photolithography



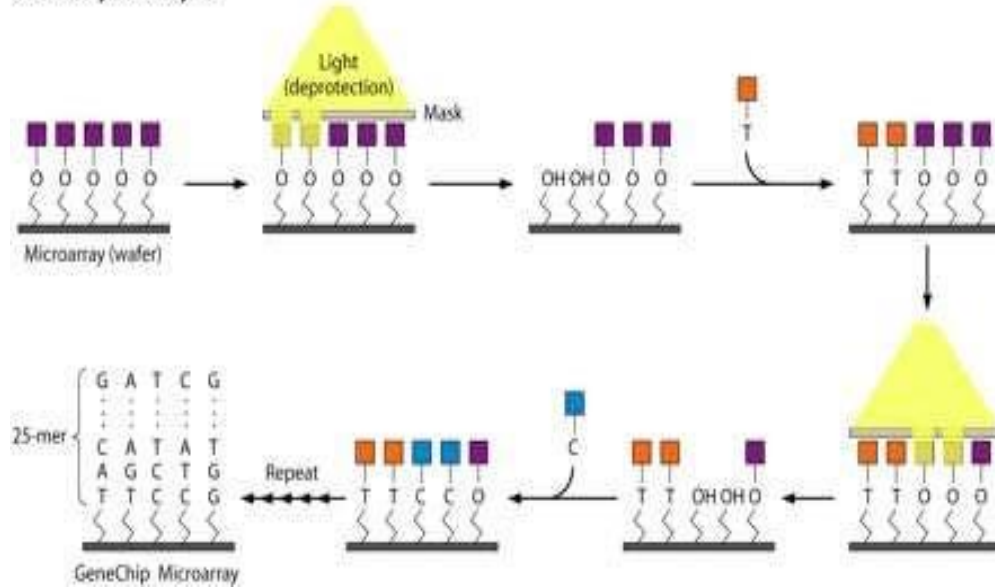
Printing of High-density oligonucleotide microarrays

Photodeprotection using masks:
Affymetrix (Photolithography)

Photodeprotection without masks:
Nimblegen, Febit

Inkjet Technology: Agilent, Oxford Gene Technology.

Chemical Synthesis Cycle



Post Processing of slides:

- DNA is usually cross-linked to the glass slides by treating with UV light or baking at 80°C, and residual amines are blocked by reaction with succinic anhydride.
- As a final step, a proportion of the deposited DNA is rendered into single stranded form available for hybridisation by heat denaturation.
- The printed slides are stored desiccated at room temperature, in the dark, until required for experimentation.

2. SAMPLE PREPARATION

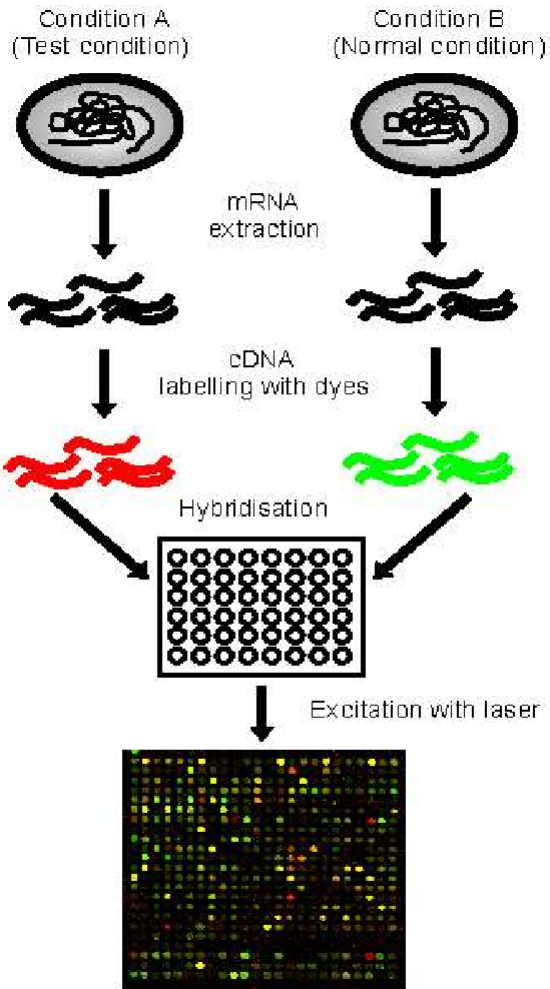
In sample preparation, RNA from the host organism is isolated, converted to cDNA and labelled with dyes before hybridization to the array.

1. RNA extraction from the tissue of interest.

- Quantity, and integrity of the total or mRNA used is important
- Numerous methods for RNA isolation available:
 - Use of Trizol®, and other phenol-based methods
 - Commercially available RNA isolation kits (eg: Ambion, Qiagen, and Promega.) provide rapid and reliable RNA extraction.

2. cDNA production: convert the RNA into a labelled form for hybridization.

- This most typically involves a reverse transcription step.



Labelling

- Combination of **Cy3 (excited by green laser)** and **Cy5 (excited by red laser)** has been used most frequently.
 - Relatively stable in light
 - Incorporated efficiently into cDNA
 - Wide separation in excitation and emission spectra
- 2 samples are hybridised to the arrays, one labelled with each dye, allows simultaneous measurement of both samples.
- 2 widely used methods of labelling cDNA:
- **Direct & Indirect labelling**

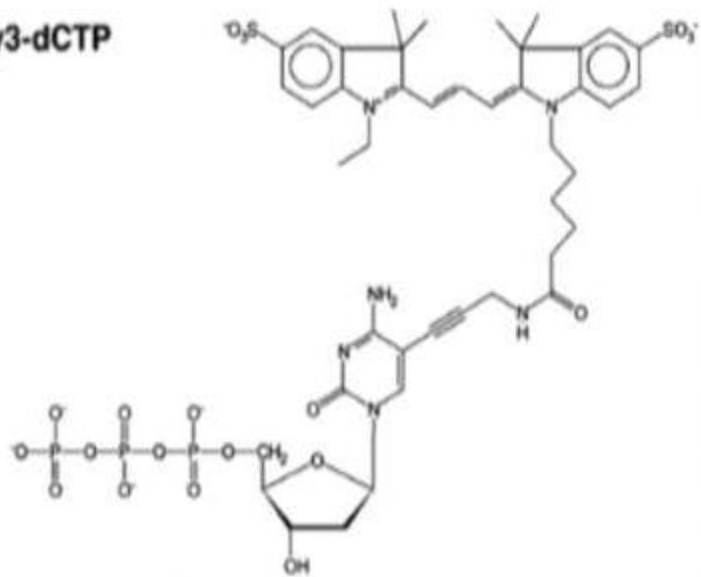
Direct labelling:

- A Dye conjugated ntd incorporated directly into cDNA by RT enzyme.
- By using dNTPs that have dye molecule directly coupled to the base, with cyanine 3-dCTP (Cy3-dCTP) and cyanine 5-dCTP (Cy5-dCTP).
- Conjugates of the Alexa dyes, **Alexa555** and **Alexa647**, the spectral analogues, fluorescent and photo-stable and are therefore the most commonly used alternatives.

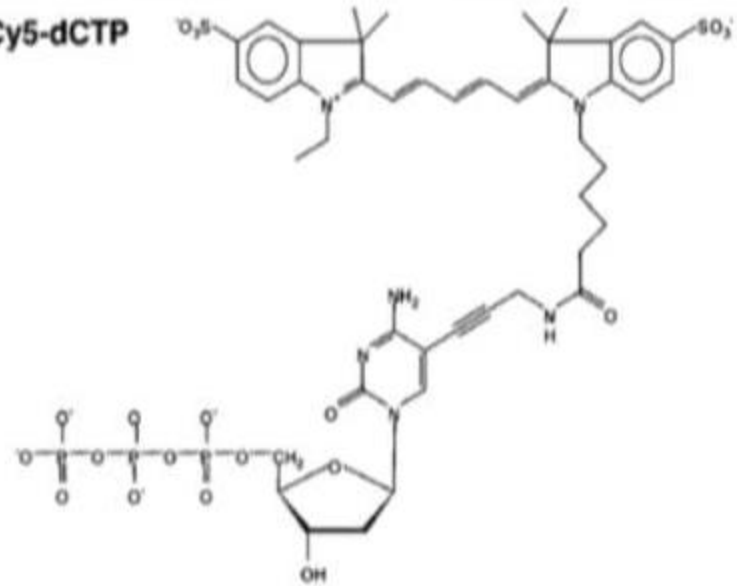
Ad: Quick and simple, requiring relatively few steps, and therefore are easy to scale up for high throughput.

Disad: Require high amount of RNA (approx 25–100 mg total RNA) for labelling reaction. The bulky dye-coupled nucleotides reduce the efficiency of the reverse transcriptase and lead to dye bias.

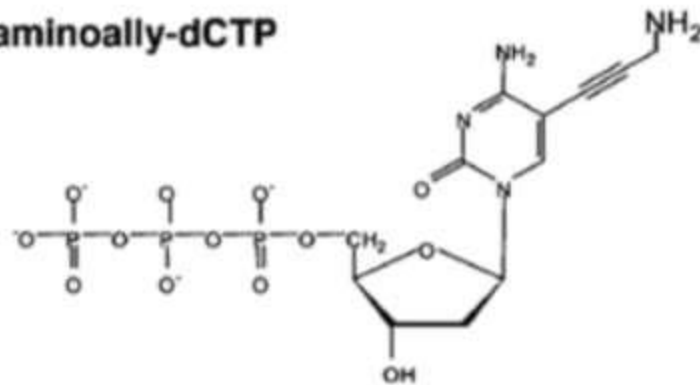
Cy3-dCTP



Cy5-dCTP



aminoally-dCTP



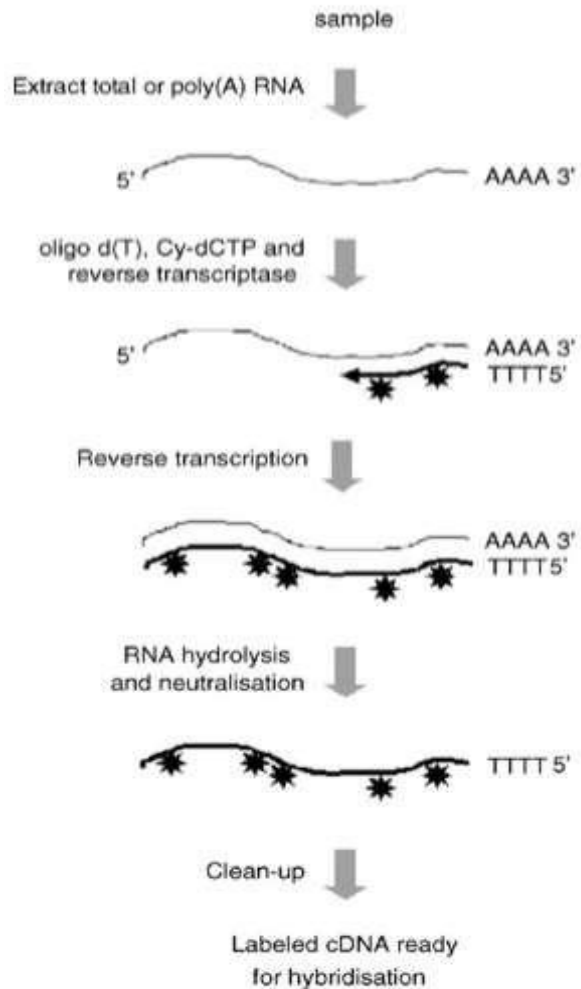
Indirect labelling:

- The RT enzyme incorporates an amino-allyl dNTP into the cDNA instead of Cy-dCTP.
 1. Aminoallyl-dNTP is added to ntd mix in the RT reaction to produce first strand cDNA
 2. After first strand synthesis, an amine-reactive Cy dye is chemically coupled to the aminoallyl groups, thus labelling the cDNA.

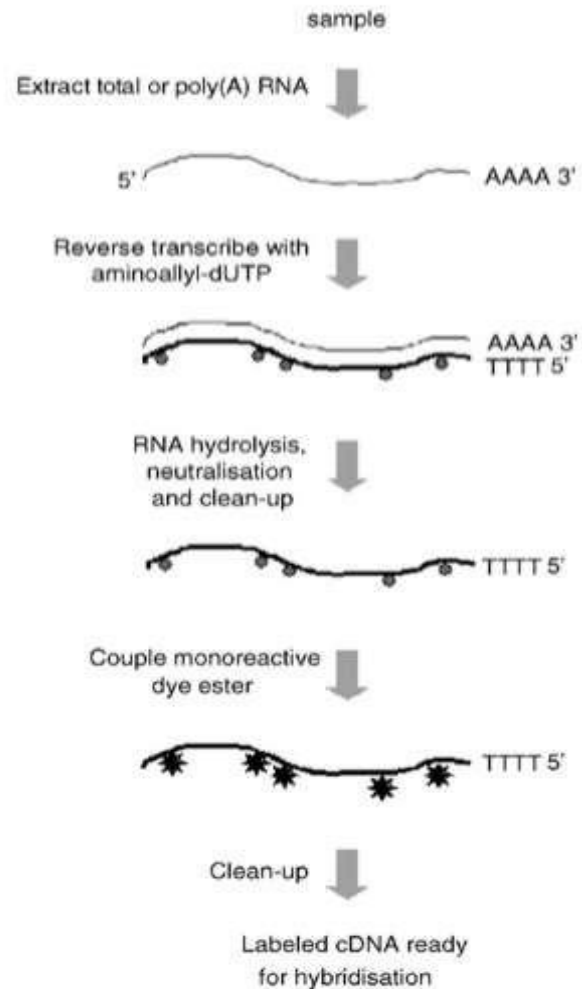
The CyDyes have NHS (N-Hydroxysuccinimide) esters that react with the aminoallyl groups of the cDNA.

- Ad: Less steric hindrance by smaller group (an aminoallyl-dNTP).

Direct labeling



Indirect labeling



Other Labelling Methods:

The sample RNAs hybridized to the complementary probes on the array are detected by **incubating the array in a colloidal gold solution.**

- The +vely charged gold particles are attracted to -vely charged phosphate groups in the backbone of the target, resulting in precipitation of nano-gold particles.
- The amount of precipitation proportional to the amount of bound target RNA.
- Ad: Instead of an expensive confocal scanner, a relatively inexpensive flatbed scanner can used to detect the gold precipitate.

3. MICROARRAY HYBRIDISATION

During the hybridization reaction, labelled targets interact with the tethered probes due to sequence complementarity.

- Appropriate hybridization conditions are critical to ensure correct measurement.
- The hybridization procedure involves several steps:
 - The arrays are blocked to minimize background.
 - The labelled target is added to the array at a specific temperature to allow complementary sequences to anneal.
 - The arrays are washed to remove unbound or weakly hybridizing material.

Blocking

- Before hybridization, the array is treated to **prevent nonspecific interactions** between the nucleic acid in the labelled sample and the array surface.
- Different blocking methods have been described by the chemistry of slide coating.
- After blocking, double stranded DNA arrays are boiled to denature the DNA and thus enhance their availability for hybridization.

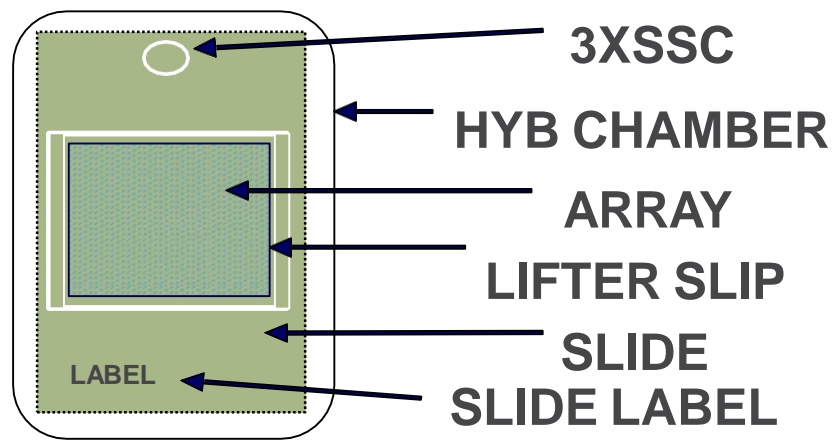
polyL-lysine arrays need to have exposed amines blocked to prevent binding of labelled material.

Achieved with a mixture of succinic anhydride, 1,2-methyl pyrrolidinone and sodium borate. Succinic anhydride reacts with and caps the amines before the excess DNA from the printed probes leaches from the spot area and binds nearby₂₁ exposed lysines.

Hybridization

Hybridization depends on the ability of the labelled target to anneal to a complementary probe strand tethered to the array.

- This occurs just below T_m of the target–probe duplex.
- The main factors affecting are temperature, pH, monovalent cation concentration and the presence of organic solvents.
- **Hybridization solution:** Contain a high concentration of salts, detergents, accelerants, and buffering agents. The most common components of solutions are:
 - Sodium chloride and sodium citrate (SSC)
 - Formamide and dithiothreitol (DTT)
 - Dextran sulfate
 - EDTA
 - Sonicated salmon sperm DNA, polyA, Denhardt's solution



After-Hybridisation Washing:

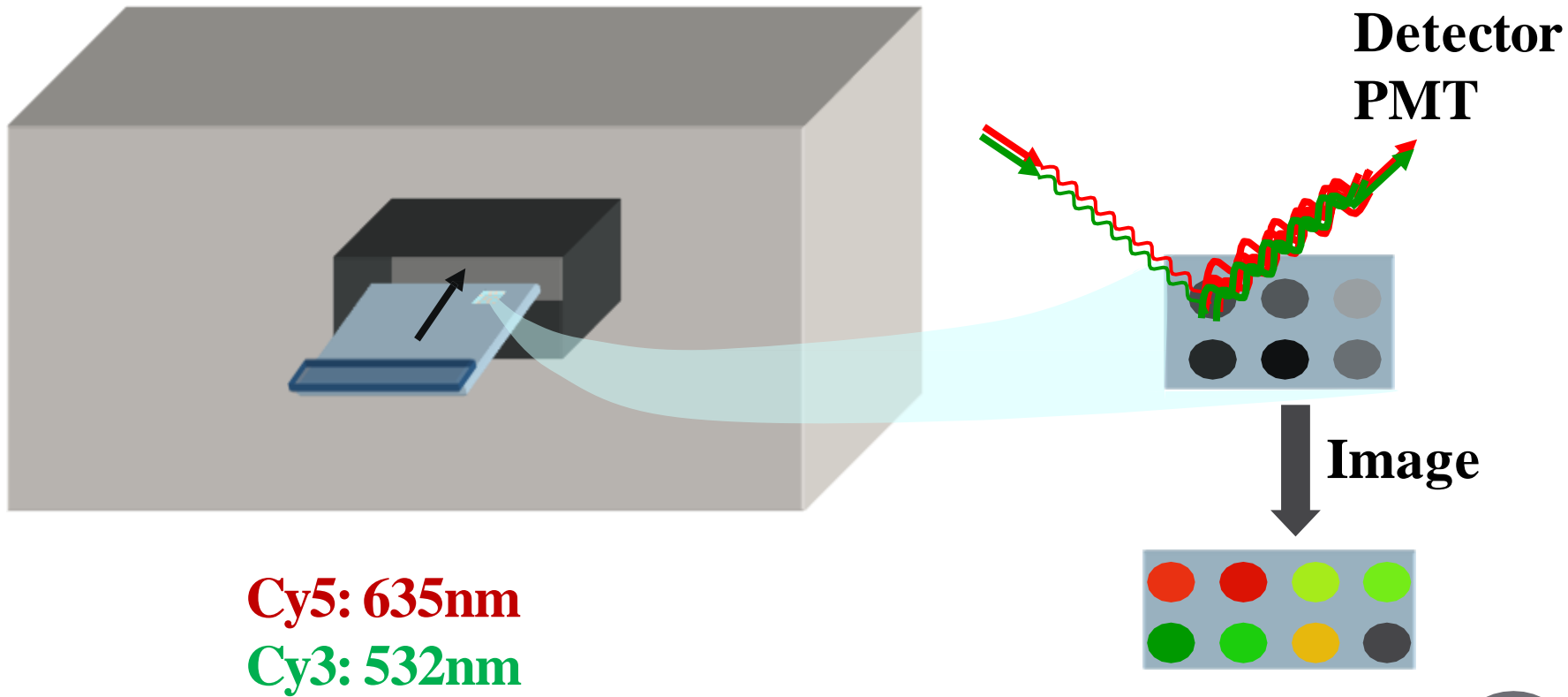
- To remove unbound target and any target loosely bound to imperfectly matched sequences.
- For good quality arrays, it is essential that both hybridization and washing is uniform across the array and that the surface is evenly dried before scanning.

4. DATA ACQUISITION & ANALYSIS

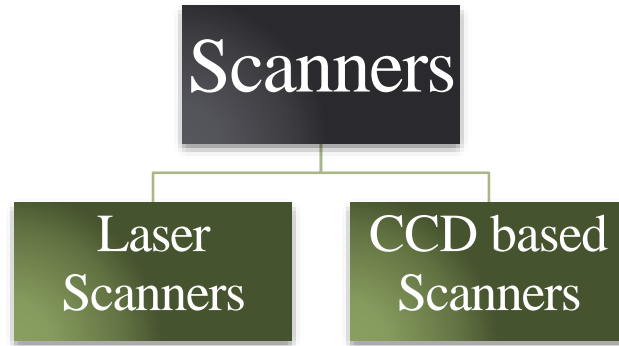
- Gene expression levels are evaluated by measuring the amount of reference and test probe that binds to each arrayed cDNA.
- Fluorescence is detected on arrays by means of a **scanner or reader** and saved as a digital image.
- These data are then imported into software that converts the fluorescence into pixels that can be counted.
- Following background subtraction and normalisation, expression ratios can be calculated.

1. Scanning

- A microarray scanner uses a light source to excite the fluorophores present on the sample molecules and then detects the emitted light, which is most typically stored as a 16-bit tiff (tagged image format) files for each wavelength scanned.
- Each image is composed of a matrix of pixels where each pixel represents the fluorescence intensity of a small area of the array.
- This digital image represents the ‘raw data’ from which the fluorescent signal of each array element will subsequently be quantified and the expression level of each target inferred.

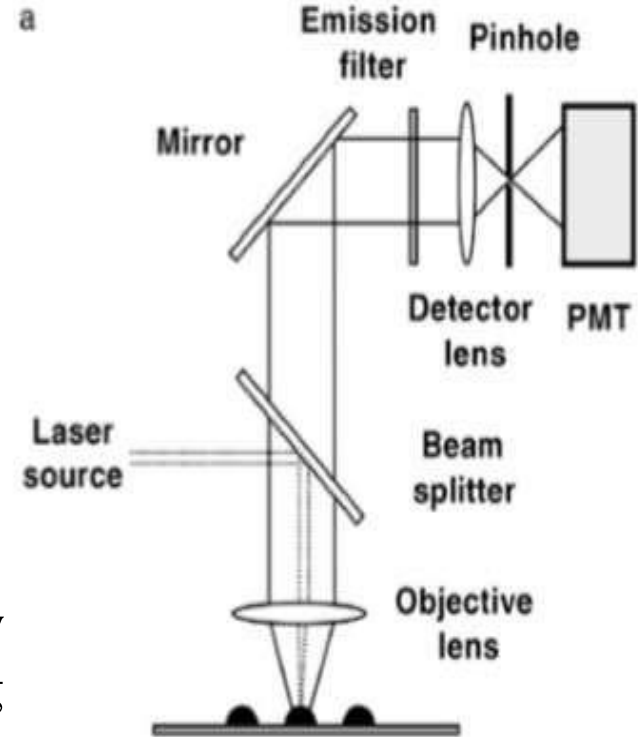


- Scanners, according to the type of excitation and detection technology they utilize.



Laser Scanners:

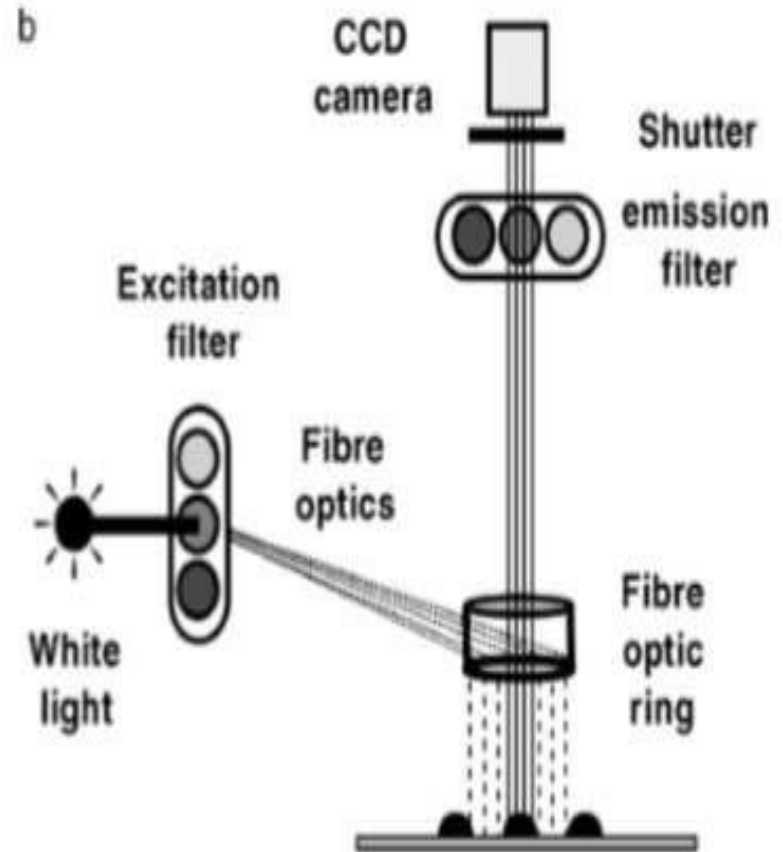
- Use narrowband laser illumination to excite fluorophores and capture the resulting fluorescence with a PMT detector.
- Any slight movement in the position of the array or difference in the starting point of the scanning head can lead to mis-registration.



CCD (charge-coupled device) Scanners:

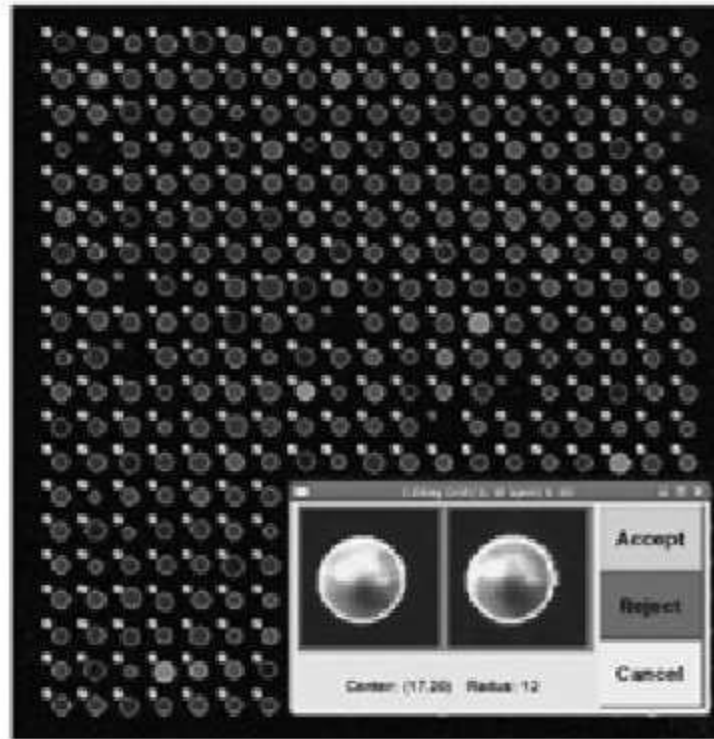
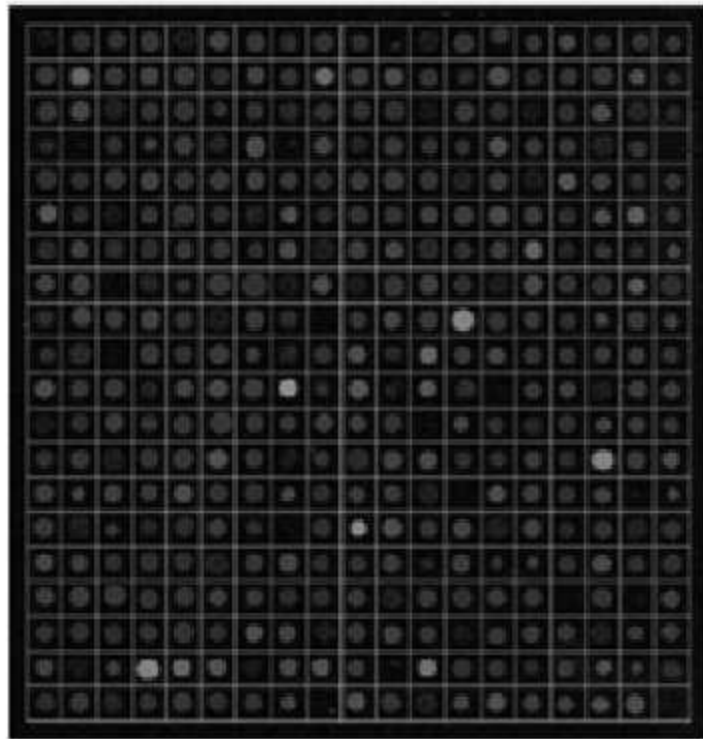
- The excitation source for a CCD detector is broad-spectrum white light, (xenon or mercury lamp), which is filtered to select the excitation wavelength.
- The filtered light illuminates a large area of the array and the fluorescent emission from the entire field of view is collected by a stationary CCD.
- A CCD chip is an array of semiconductor devices, or camera pixels, where each camera pixel stores an electrical charge generated by light from the emission filter.
- This charge is proportional to the intensity of the light, or number of photons, that reaches the semiconductor.
- Electronic circuitry on the camera converts pixel electron counts from the CCD chip into a digital signal that represents the intensity of each pixel.

- CCD scanners tend to capture multiple images from different areas of the array and then stitch them together to create a single image.
- Disadvantage: Any imprecision in the stitching, or photo bleaching due to repeated exposure of overlapping regions, will result in inaccurate and uneven signal quantification.
- Advantage: Data from all wavelengths simultaneously collected and therefore no mis-registration issues.



2. Image Data Generation:

- The images obtained from the scanner are imported onto software that converts the 2 scans into pseudo-coloured images that can merged.
- Software for this purpose is available from a no. of sources, both academic and commercial.
 - Gridding
 - Background sampling
 - Target intensity extraction
 - Signal filtering
 - Ratio Calculation
 - Processing expression data



Example of automated gridding and spotfinding by the Dapple software

2.3. Target intensity extraction:

- To determine spot intensity, the mean intensity of the pixels contained within the spot circle are determined for corresponding Cy5 and Cy3 image.
- Subtraction of background pixels for the corresponding element then yields a net intensity value for the particular arrayed DNA.

4. Signal filtering:

- Pixel intensities for unexpressed or poorly expressed genes would be expected to be close to the background intensity of Cy3 and Cy5 image.
- To distinguish such genes, data may be filtered so that only those genes expressed above a certain level are analysed further.
- Typically spot intensities that are less than 2 fold above background can be excluded.

2.5. Ratio Calculation:

- Calculate the ratio of the background corrected intensity for the Cy5 image to that for the Cy3 image for each spot.
- Accuracy of this measurement can vary, in particular on arrays with high background.

$$M = \log_2 R/G = \log_2 R - \log_2 G$$

M < 0, gene is over-expressed in green-labeled sample compared to red-labeled sample.

M = 0, gene is equally expressed in both samples.

M > 0, gene is over-expressed in red-labeled sample compared to green-labeled sample.

2.6. Processing expression data:

- The ratios are normalised to exclude any bias towards one of the two probes.
- Normalization is carried out based on the assumption that only a small proportion of genes will be differentially expressed among the thousands of genes present in the array and/or that there is symmetry in the up- and down-regulation of genes.
- Normalised data can then be ranked to identify expression changes between the reference and test condition.
- Finally data can be organised to identify similar expression patterns.

TABLE 1. Comparison of microarray platforms^a

Microarray	Principle(s)	Format(s)	Density	Relative cost	Diagnostic application(s)
Printed	Glass slides are used as the solid support for printing DNA probes	For dsDNA, PCR amplicons (200–800 bp) from known genomic sequence, shotgun library clones, or cDNA are used; for oligonucleotides, 25–80-bp probes are synthesized	Moderate (~10,000–30,000)	\$\$\$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance
In situ synthesized	Oligonucleotides are synthesized directly on the surface of a quartz wafer using photochemistry; multiple probe sets (one perfect-match probe and one mismatch probe) are included per target	Affymetrix GeneChips, 20–25-bp probes; Roche NimbleGen, 60–100-bp probes; Agilent, 60-bp probes	High (Affymetrix, >10 ⁶ ; NimbleGen and Agilent, 15,000–>10 ⁶)	\$\$\$\$	No commercially available applications; pathogen detection and identification, antimicrobial resistance detection, viral discovery, molecular surveillance, strain typing
High-density bead arrays	Sequence-tagged beads are randomly assorted onto fiber-optic bundles or silicon slides	SAM, 96 samples; Sentrix BeadChip; 1–16 samples	High (~50,000–10 ⁶)	\$\$\$	No commercially available applications; potential use in microbiology but no studies published to date
Electronic	Electric fields are used to promote active hybridization of nucleic acids on a microelectronic device; streptavidin-biotin bonds immobilize the probes on the array surface	NanoChip 400; capture probe down; amplicon down; sandwich assays	Low (400 max)	\$\$	Commercially available products discontinued; pathogen detection and identification
Liquid-bead suspension	Spectrally unique microspheres provide solid support for application of probes or universal sequence tags; bead hybridization with fluorescently labeled target DNA is measured using flow cytometry	Direct DNA hybridization; competitive DNA hybridization; solution-based chemistries (ASPE/TSPE, OLA, SBCE)	Low (100 max)	\$\$	FDA-cleared xTAG RVP assay; pathogen detection and identification, antimicrobial resistance detection, strain typing

^a Data from reference 135. \$\$, low-moderate cost; \$\$\$, moderate cost; \$\$\$\$, high cost.

THANK YOU